

6 May 1982

How not to teach engineers

Changing the name of a research council will not help British engineering out of the rut. But what will?

British engineering education is once more in turmoil. In spite of the appearance of the Finniston committee's report on engineering education just over two years ago, which went a long way to meeting previous discontents, and a more recent decision by the Science Research Council to add engineering to its formal title, the engineering professors from British universities, long known as a body of turbulent men, are still pressing for a separate engineering research council just for them. The argument that inspires this movement has little substance and indeed is illogical if taken seriously. What the engineering professors are doing is to combine elements from the Finniston report and their own practice which are consistent with each other but inconsistent with the needs of potential engineers, with the needs of British industry as they should be and indeed with the needs of education.

The Science Research Council's decision to call itself the Science and Engineering Research Council seemed last year to be strictly cosmetic. In reality, the Science Research Council had for many years been trying with conspicuous success to increase the proportion of the funds spent on engineering research in British universities. The fact that over the years it has found this task very difficult is as much an indication of the receptiveness of the market — potential researchers in universities — to the availability of research funds as a sign that engineering is included in comparison with science and other fields of enquiry. For the principle is that research is almost inevitably a less important part of British engineering education, at least as at present constituted, than of other scientific fields of enquiry (medicine perhaps excepted). For one thing (as the Finniston committee noted) many of those who enter engineering courses do so because they wish to join some part of the labour force at an early stage — wish to "get something done". Second, engineering research frequently entails use of large and expensive experimental rigs which, at least as British universities are at present organized, hard to come by. Third, it is by no means clear whether research in a formal academic sense contributes to the creativity which the engineering professors, like the Finniston committee, consider to be the element in British engineering now most in need of being fostered. In short, the case for regarding research as an essential part of engineering education rests only on the proper assumption that people who teach and do nothing else are probably poor teachers. Yet, underpaid as they are, many British engineering academics prefer to spend what spare energy they have, not on the preparation of formal research reports, but on activities that may in the long run be just as useful to them as teachers — consulting for companies, working on real projects or perhaps even writing books that simplify scraps of information already known.

So why is the cry that more funds should somehow be channelled into academic engineering research so insistent? The chief answer seems to be that the engineering professors and their followers have noted how, in other fields of science, people's

prestige or even promotion depends on their formal completion of research projects of various kinds. Finniston was persuaded of the importance of this model, suggesting that engineering would not acquire the status he rightly wished to give it until most engineering academics were able to brandish *curricula vitae* stuffed with research papers published in some formal body of literature. The argument may be fair. If this were indeed what most engineering academics could boast of, they would no doubt walk taller when mixing with their scientific colleagues in universities. But it does not follow that the condition of engineering education would thereby be improved. Indeed the Finniston report itself drew attention to the importance that engineering academics should be people who, first of all, were able to win the respect of their industrial rather than academic counterparts. For, the argument goes, only then will their students respect them and listen to what they have to say.

The same depressing conclusion is not equally applicable elsewhere, in many of the countries of Western Europe and the United States for example. The reason for that difference, however, lies in one feature of the British engineering curriculum which needs more attention than it was given even by the Finniston committee. Starting from its assumption about the motives persuading undergraduates to enter engineering courses in British universities, the Finniston committee argued that people wishing to work as engineers should be given plenty of opportunity to do so quickly. In other words, the more a student was persuaded to acquire a good understanding of physical (and these days biological) science, the more likely he was to lose his sense that engineering is something different. The result of that argument, accepted by the Finniston committee and most of those enthusiastic about its conclusions, is that even those engineering courses at British universities which have been "enriched" differ very little from the older courses to which have been grafted elements of the curriculum concerned with law, industrial relations and production engineering (whatever that may be). This implies that even if, as is likely, the next few years see a spread of these enriched courses throughout the British university system, engineers leaving the universities may still be ignorant of the rudiments of the sciences to which their skills apply. Is this a recipe for providing the kinds of engineers likely to be at the forefront of industrial innovation? Or is it a device for making sure that even if the other obstacles to change in British industry were removed, engineers would still feel trapped by the past?

The Finniston report, rich as it was in comparisons between the economics of Britain and its industrial competitors, seems not fully to have heeded the implications of the curricular comparisons that it also included. Elsewhere, especially in Western Europe, engineering education is indeed an intimate amalgam of sciences and its application. To be sure courses are, by British standards, long — five rather than three or four years are commonplace. The graduates from these more rigorous engineering schools appear to make a more direct contribution to the innovation of products and processes. The Japanese example is perhaps even more striking — university engineers tend to be well trained in basic sciences and the companies which eventually employ them shoulder much of the task of helping them understand how their knowledge can be used. Why should not the British educational system follow one or other of these paths? It

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would be wrong to blame the engineering professors, who appear willing to change, although they contribute to the suspicion growing between science and engineering in Britain. Nor is the government to blame, for it seems fully aware that British engineering should be more innovative and more flexible. The real difficulty is that for industry survival must now depend on making saleable products for the first time, and British industry does not fully understand what skilled engineers could accomplish.

The Finniston report itself provided plenty of evidence to show that British engineers once at work have ahead of them a poor prospect for a career. A few years after formal graduation, and once the two-year period required to become a chartered engineer is completed, the salaries of those still working as engineers for British companies appear to level off. While the statistics are notoriously untrustworthy — many who began as engineers may quickly become salesmen or administrators — industrial companies appear to set little store by the work required of professional and experienced engineers. But especially now, when the new technologies in microelectronics and telecommunications have thrown up almost an embarrassment of devices that might be manufactured and sold profitably, and when one of the most serious problems facing British companies and their competitors is to decide which of these might be made cheaply and sold profitably, there is a greater than ever need of people with a proper engineering education who might help chart a reliable course for the future. What this in turn implies is that British industry must look for a system of higher education in engineering that will produce large numbers of qualified and creative people who will more often be employed in research and development than in the running of plants, production engineering or whatever and, if the engineering professors would reflect on that ideal state of affairs, they would surely recognize that then the status of engineers in Britain would truly be enhanced. Contributing to the health of the economy would automatically win the respect that could not be acquired by a list of research papers an arm long. It might then even be the case that engineering teachers, themselves transformed by industry's expectations of engineering qualifications, would more eagerly take up the funds that the Science and Engineering Research Council is apparently eager to seize back. But another research council, exclusively concerned with engineering research would be at best a red herring.

Economic research?

Can Congress realize its adage that investment in basic research will aid economic growth?

The US Congress is now wrestling with a problem common to all the industrial democracies: how to get the most economic benefit out of the nation's investment in research and development. Because the US economy is so decentralized, Congress is attacking the problem with the only central mechanism under its control, the federal research budget worked out each year between the current Administration and Congress. Last December the US Congress, on a sweeping 90 to 0 vote, decided that one way to do this was to set aside one per cent of federal research and development funds (excluding those going to in-house research) for small independently-owned companies employing 500 people or less. These firms have traditionally brought forth many more innovations than the giant US corporations that gobble up most of the federal industry research and development budget and spend most of the private money spent on industrial research in the United States.

The "set-aside" is meant to do two things: first to establish a federal policy that enhances the role of small businesses in research and development, second to generate more research results that can be turned into commercial activity on the open market. The plan is modelled on a successful programme developed by the National Science Foundation that has enabled small firms to attract private capital, increase employment and, in a few cases, achieve great commercial success.

Other countries are also trying to address this problem. Great

Britain has two smallish programmes, one that sets up "teaching companies", or long-term collaborations between universities and industry, and the CASE programme that works at the student level, placing individual students in companies in such a fashion that their industrial research earns them credit towards their degree. Although having some direct federal programmes in priority areas, West Germany relies primarily on tax incentives to stimulate industrial research and development. Small German firms tend to contract with larger ones for the basic research they need to do before a project reaches the commercial stage. All are concerned with the same thing: how to transfer knowledge out of the basic research sector and into the increasingly competitive national and international marketplace.

However, representatives of US universities have objected strongly to the bill, arguing that while the objectives are worthy, the mechanism of "set-asides" will shrink the share of the federal research budget that goes to the universities. They would not care if Congress was in a spending mood and simply authorized more funds for small research and development firms. But everyone in Washington is tight-fisted these days. The Administration could not have supported the legislation, as it does now, if it proposed to add more money to the budget. The legislation proposes to take the money from the existing budget, and so spokesmen for the universities have been crying, "Why us?", although the Senate version, which the House will probably also pass, limits the "raid" on basic research funds to one per cent of the basic research funds of any agency. The objections from university spokesman are loud and clear. However, there have been fewer objections from the contractors and applied researchers who now get the remainder of the federal research and development budget, some of whose funds will be used to make up the balance of the new programme.

University scientists have reason to be concerned about protecting their share of the federal research and development pie from encroachments. But the objections to this tiny set-aside — which is likely to be \$300 million in all, of which a small part would come from basic research — have been strident even at a time when the workers at General Motors accepted voluntary wage and benefit cuts to help bail out that ailing enterprise. If the most entrenched workmen's unions in the United States can make concessions to remedy the US economic troubles, cannot the better paid, supposedly more high-minded scientists?

In objecting to the legislation, spokesmen for universities and for basic research have been anxious to preserve their own turf at the expense of everyone else's. Senator Warren Rudman, the prime mover behind the bill, is fond of quoting a prominent scientist at Massachusetts General Hospital stating he was not concerned about whether small businesses got more research and development, only about whether "one" of his investigators lost a grant because of it. What is missing here is any perspective from the scientists that they are part of any larger effort. Remember the old idea that the United States should invest in basic research for the sake of economic growth, productivity and the creation of jobs?

The new bills, therefore, put the US university science community squarely on the spot. Thirty-seven years ago, Vannevar Bush and his colleagues persuaded the US government to sponsor basic research in the universities, and required that it have special, protected status, its own programmes and agencies, because, as Bush wrote, "Basic research leads to new knowledge. It creates scientific capital. It creates the fund from which the practical applications of knowledge must be drawn".

Small firms are also part of the "fund" from which practical applications are drawn, both those which do basic research and those which do not. If the university spokesmen are truly concerned about national productivity, creating new inventions, and economic health — as they have said they were in selling their own budget requests to Congress in the past — they should support any measures that further that goal. At least they should offer constructive alternatives. But they do the image of science — and the US economy — no good by limiting their comment to paranoid fears that their particular sector will be hurt.

National labs reach crossroads

US report looks abroad for ideas

Washington

The Energy Research Advisory Board (ERAB) of the US government has issued its preliminary advice on a critical study of the fate of the Department of Energy's (DoE) national laboratories. This is a key study because the laboratories are also being looked at by the White House, which may not be favourably inclined towards them and will have much to say about their future.

At stake is the role of nine facilities (see below), some of which do the most intensive high technology research in the world — on atomic weapons, fusion and lasers — gobbling up \$2,300 million, or half of DoE's annual research and development budget each year. Apart from their atomic weapons work, they are entirely the creatures of DoE. Starkly put, the choice is whether the laboratories should throw their doors open to become national centres, performing research for other federal departments, private industry, perhaps even other countries, or whether, as budgets decline, they should concentrate on a few things they do well and consolidate, with some of them closing down.

The debate will be joined in the autumn when the ERAB panel makes a final report. At that time, the White House Science Advisory Committee will be studying the fate of the DoE laboratories and those of the Department of Defense. Mr George A. Keyworth II, the President's science adviser and an alumnus of Los Alamos, has said that he thinks some of the laboratories should close down. Last December, the Administration proposed that all DoE research be moved to the Department of Commerce, a suggestion that no one in Washington now takes seriously. And James Edwards, Secretary of DoE, surprised ERAB recently when he told them that, in his view, the laboratories should remain in existence and throw their doors open to become national technical centres. So the discussion, once joined, will be lively.

The ERAB panel is headed by Ivan Bennett, dean and provost of New York University Medical Center. DoE requested the study last September. A preliminary report was sought this spring to feed into the White House's review. Although the group has not decided its own view of the laboratories' fate, the preliminary report outlines the laboratories' strengths and future options.

Some of the institutions, like Los

Alamos, were founded as part of the weapons programme and retain important roles in weapons research. Others, such as Ames Research Laboratory run by the University of Iowa for DoE, do basic research. Oak Ridge has undergone a major transformation into a general applied research laboratory since its beginnings during the Manhattan project.

The appendix to the ERAB panel's report deals with how major laboratories in other countries have adapted to changing times or to the stark fact of having fulfilled their mission. The UK Atomic Energy

mission and that such organizations can indeed change their mission without loss of vitality."

Other examples from abroad are Amersham International, the UK government's source of radioisotopes which has been spun off as a company, and France, where government laboratories commonly create companies to perform specific work for industry. The report notes the Dutch pattern of numerous small laboratories, which are two-thirds government-sponsored and one-third sponsored by industry.

Sweden's Studsvik Energiteknik AB, the government's energy laboratory, had three years' warning that its government funding would be cut by 70 per cent. The three years enabled the group to make a smooth transition to becoming predominantly sponsored by outside interests.

The implication for the US national laboratories is that they can adapt and so probably should.

The carefully worded list of options presented by ERAB includes: "concentrating" the laboratories' efforts through vigorous management by the DoE; freeing them of legal constraints so they can work for other sponsors; transferring other federal tasks to them, making them more multi-faceted or narrowing their mission "to correspond with resources allotted"; closing one or more laboratories, or mergers. The suggestions are intriguing, but to implement any one of them will be a gargantuan task.

Deborah Shapley

National laboratories covered in the ERAB report

Ames Laboratory
Argonne National Laboratory
Brookhaven National Laboratory
Los Alamos National Laboratory
Lawrence Berkeley Laboratory
Lawrence Livermore National Laboratory
Oak Ridge National Laboratory
Pacific Northwest Laboratory
Sandia Laboratories

Authority's laboratories at Harwell are discussed at some length. Founded in 1946 to assist in developing civilian nuclear power, Harwell's task was nearly complete by the mid-1960s. Then, the report notes, Harwell kept the same disciplines but took on new clients outside the government's agency. The lesson of Harwell, it states, is that "once a large laboratory has been assembled, it can be useful to government in fields that extend far beyond its original

Disarray on chemicals control

Brussels

Fears are growing in environmental circles that the special programme on the control of potentially hazardous chemicals set up by the Paris-based Organization for Economic Cooperation and Development (OECD), is running into difficulties as a result of the Reagan Administration's pro-industry and protectionist policies. The downgrading of the US Environmental Protection Agency (EPA) has coincided with a loose implementation of the toxic substances control act (TOSCA). Jacqueline Warren of the Natural Resources Defence Council, a leading environmental pressure group, has cited EPA figures on the premarketing notifications for new chemicals submitted since April 1979 showing that 66 per cent of submitted notices failed to include toxicity data, thus undermining progress to establish procedures to reduce the risk of new chemicals.

Officials at OECD's headquarters are cagey, in view of the United States'

negotiating weight, about admitting allegations that the enthusiasm evident under the Carter Administration for the OECD programme has waned. A legislative gulf has certainly grown between the United States and the EEC, as a result of the way that TOSCA is being implemented and the changes within the EEC after the Sixth Amendment came into force last September. This is likely to impair prospects for rapid agreement within OECD.

Under the Sixth Amendment of the 1967 directive on dangerous substances companies must submit a stringent pre-marketing dossier on a new substance not only to national authorities but also to the European Commission and to the other member states. Not surprisingly, EEC countries are now disappointed that disagreements within the OECD still loom large in two crucial areas which appear to have been resolved by the 6th Amendment. In October this year a high level OECD meeting will be tackling the areas in

question — how to ensure satisfactory quality controls for the implementation of the "Good Laboratory Practices" scheme agreed on last year, and how the confidentiality of data is to be guaranteed.

The Good Laboratory Practices scheme represents a commonly applied definition of the standards and procedures to be used in laboratories testing new chemicals. Unfortunately mistrust has clouded the agreement. High standards cost money and governments are not convinced that everyone is abiding by the rules as strictly as they ought to be, so the October meeting will examine proposals for methods to check that the practices are properly upheld.

This will be another small step towards the programme's ultimate aim of guaranteeing that the marketing authorizations handed out nationally are acceptable elsewhere. Only by using the Good Laboratory Practices scheme can a standard dossier of data on a new chemical substance, the minimum pre-marketing data, be accepted by other national authorities.

The scheme involves, though, a free flow of allegedly valuable commercial information, and this is causing worries in the chemical industry that such information will not remain confidential. The issue is complicated by the fact that there are large differences among OECD countries in the laws relating to freedom of information. Environmentalists are equally worried that poor access to information on toxicity considered confidential by industry will violate the principle of public participation in the assessment of the hazards of chemicals to which the public are exposed.

The OECD's agreement on exactly what information is relevant to hazard assessment has disappointed both the industrial and environmentalist lobbies. The various OECD working groups have come up with a document which defines what data should not be considered confidential and which specifically excludes information such as spectroscopic data which might reveal the chemical identity of a substance before a manufacturer wishes it to be generally known. A final agreement has yet to be reached in sensitive areas such as pharmaceuticals. The United States is held to be particularly responsible for back-tracking on this question as the list of non-confidential data continues to be reduced. If too much remains confidential, hazard assessment made by industry or government will be harder to question, especially if there is consistency on marketing authorizations.

So it seems there is still considerable work to be done within OECD before guidelines are agreed on what hazards can be considered acceptable, and this may prove to be the biggest stumbling block to further international cooperation in this field.

Jasper Becker

How Dr Melamed lost his degree

In 1976, new regulations came into force in the Soviet Union governing VAK, the "Higher Attestation Committee" responsible for conferring the degrees of Candidate (PhD) and Doctor (DSc/DLitt). The full text was not published at that time (a booklet was promised for "later") but the media placed considerable stress on the fact that the new regulations demanded scrutiny of the political attitude of the postulant for the degree.

It now appears that this requirement can work retroactively. During the past year, at least seven Jewish scholars have been deprived of their degrees, for having exhibited "anti-patriotic" attitudes in applying to emigrate to Israel. A document — which claims to be a transcript of such a deprivation process — has just reached *Nature*, and is reproduced here in a slightly abridged form.

Vera Rich

From the stenographic transcript of the Scientific Council of the Faculty of Geology of Moscow State University, 20 January 1982.

Chairman (Professor Adonis G. Gayanov): Members of the board already know the agenda for today's session: *inter alia* the stripping of Vladimir Grigor'evich Melamed of his academic titles due to his anti-patriotic dealings, unworthy of a Soviet scientist.

Melamed is not present, he is ill. On 18 January, two days before this session, he notified me that he had a sickness certificate (*reads Dr Melamed's notification*).

I feel it is particularly necessary to point out the problem of his absence for the following reason. According to paragraph 5 of the regulations of VAK on the conduct of sessions for the stripping of academic titles, the person from whom the title is to be stripped has to be present.

(*After some discussion, — including pressure from the public gallery — it was decided to proceed in Melamed's absence*). *A.G. Lyubimov:* Allow me to read the material from the special committee of the board, set up to investigate the activities of V.G. Melamed, who expressed the desire to emigrate to the state of Israel:

Melamed has been working at Moscow State University for approximately thirty years. Lately he was in charge of one of the laboratories, and was dealing with pressure-modification of the permafrost process. He distinguished himself by his energetic attitude and was therefore admitted to the Party. In 1975, he received the degree of DSc, specializing in geophysics.

In November 1980, he submitted a request to emigrate to the state of Israel, which is known to be capitalistic and no friend of the Soviet Union. In view of the increasing severity of the international situation and all that has been stated previously, we find that the decision of Melamed is an anti-patriotic act, unworthy of a Soviet scientist.

We are of the opinion that Article 104 of the VAK on the stripping of scientific titles can be applied to him. Dated 18 December 1981. Signed by all members of the committee

Chairman: Who wants to comment?

Professor Epinat'eva: I have a question about Melamed's scientific profile. Would you please specify what he has achieved in the scientific field?

Chairman: This is not the question. We are concerned with something else, not science. We are not going to discuss the

scientific work of Melamed, so this is not relevant.

Professor Dmitriev: Will the minutes of the decision of the committee be added to the documents that VAK receives from us?

Chairman: Yes.

Dmitriev: Then I want to point out an error. Melamed did not defend his doctoral thesis in 1976, as the committee says, but in 1977. And it is an ill-chosen expression to say, "and all that has been previously stated". From this one might conclude that he should be deprived of his degree, not only because of his wish to emigrate from the Soviet Union but also because he worked for Moscow State University for thirty years.

Chairman: Good, we shall correct this. Any further comments? (*Silence*). Good. I call on Arkadii Vasilevich Kalinin, member of the committee.

Kalinin: Today our board has a rather difficult task, which is, however, completely justified by VAK regulations. As you all know, Article 104 of the VAK regulations deals with the stripping of academic degrees due to conduct unworthy of a Soviet scientist. This includes offences such as anti-patriotism. This leaves our board one choice, to decide whether or not Melamed's offence can be interpreted as anti-patriotic or not.

According to the law, emigration is permitted for the sake of reunification of families. Melamed informed the departments which grant exit visas that this was his intention. However, no law prohibits public organizations from judging Melamed's activities.

Academic titles in the Soviet Union are conferred not only on the grounds of scientific criteria. . . We consider the function of an academic title to be more important than in the capitalist world. It confers not only the right to a function, but a salary. If Melamed remained a doctor, he would continue to do things to which he no longer has a right . . . His titles should be taken away from him. . .

Professor Nikitin: I should like to add something in order to show two aspects of the anti-patriotism of Melamed. First, he was a Party member for a number of years and also a propagandist. When submitting his papers applying for an exit visa, he turned his back on all his early ideas.

Second, he is a qualified specialist, a scientist. Now he is leaving in order to work on behalf of Israeli science, to work for our opponents, our enemies. And this is even more than anti-patriotism.

Chairman: Thank you. Does anyone else wish to speak? The case is probably completely closed and we can begin voting.

J. V. Medvedkov: Permit me to say something, not everything has been said. I am especially qualified to speak. Before I became involved in soil-sciences, I studied law.

It is, as you know, not only a moral judgement or the opinion of colleagues if Melamed is stripped of his academic titles . . . they give a right to a scientific function, a salary, the possibility of scientific work appropriate to his academic level, admission to libraries etc.

A case like this is treated completely unambiguously by a special juridical act. I mean the Final Act of the Helsinki Accords of 1975. I should like to quote from *Izvestiya*, 2 August 1975.

The submission of an application for reunification of family shall not lead to changes in the rights and duties of the person who made the request or of members of his family.

Hence it is inadmissible that the board should seek to limit Melamed's rights because of his wish for reunion with members of his family in Israel.

I have not heard any proof that Melamed's conduct constitutes an anti-patriotic offence.

It is no accident that in such a complete work of reference as the *Soviet Encyclopaedic Dictionary* (Moscow, 1979) the term "anti-patriotic" does not occur. One can use an expression which comes close to it, namely "anti-Sovietism". This term does occur in the *Soviet Encyclopaedic Dictionary*. On pages 63-64 it says that anti-Sovietism is an attempt to defame and misrepresent everything that the Soviet Union has achieved in economic, political and cultural fields.

When it is stated that Melamed's conduct is anti-patriotic, then he is apparently also accused of anti-Sovietism.

By whom and where has it been proved that Melamed has done anything to misrepresent and run down those things which the Soviet Union has achieved?

His application to emigrate was submitted without any publicity. It is an act which falls within existing laws and standards. He did not give public announcements and did not publish books which misrepresent and run down all that the Soviet Union has achieved . . .

I fear that in fact, the Soviet Union will be harmed by those people who have applied to the board with the demand that Melamed be punished.

Chairman: Any further comments?

Mrs F. Melamed: My husband is ill at the moment and I do not understand how it is possible for his case to be considered in

absentia . . . Today a matter is being discussed that is of vital importance to him. His entire 30 years career in the service of Soviet science has been put in doubt. Can one call such a career unpatriotic? . . . Why do members of the board disregard the signature of the Soviet Union below the Helsinki Final Act?

Professor Epinat'eva: It is one thing if a worker at a normal research institute or even the Academy of Sciences expresses the wish to emigrate, but it is totally different for people working at the university to do so. The latter are dealing with young people, aren't they? The act that Melamed performed distorts the world view of our youth.

S.A. Kats: This concerns the shattering of an entire human life. And you limit yourself to two short sentences. If the matter had been dealt with by the court, the procedure would have been very different.

Professor Kalinin: I am against the fact that "anti-patriotism" and "anti-Sovietism" are equated. When Herzen left Russia, it was an act that we call "anti-Tsarism" and not "anti-patriotism".

It was the last straw to say that Melamed would be anti-Soviet. But can we call his action complimentary to the fatherland? He did not want to leave Moscow State University in order to make life easier for us. So we do not have to make life easier for him . . . I do not think that we are in violation of the Helsinki Accords.

G.O. Marshuhuk: I myself disapprove of Melamed's action, and I told him so. But disapproving is one thing and accusing him of anti-patriotism, and punishing him severely by denying him the right to scientific work is something totally different.

I. S. Irlin: There is no doubt that Melamed behaved within the framework of the law . . . During the past six to seven years, some thirty thousand people left the Soviet Union to be reunited with their families. Among them there were quite a number with doctoral or candidate's degrees. But personally I do not know of any case where one was deprived of his scientific degree. In my opinion the board wants to create a precedent.

Professor V.V. Karus: According to Article 104 of the VAK regulations, one needs three qualities to hold a scientific title: qualification, patriotism and regard for ethical standards . . . Patriotism is love for the fatherland which raised us. But Melamed decided to leave the fatherland at a time of severe international tension and for a country that propagates a malicious hostility against the Soviet Union . . . The right to work will not be taken from him, but why should he get an extra salary for working in a scientific field?

Chairman: I request you to vote for the stripping of Melamed's degree of Candidate of Physico-Mathematical Sciences. For the motion? . . . Carried unanimously. I hereby declare the meeting closed. □

Agricultural biotechnology

Soon to start

Springtime has not induced germination of the British Technology Group's (BTG) plans for an agricultural genetics company but it is still expected to bloom this year. One snag at present seems to be the degree of scepticism among those of the calibre to be potential scientific directors about the ability of any such company to be profitable within five years, as BTG would want.

Although BTG considers the topic too sensitive to discuss, by all other accounts the plan is that the new company should have the same kind of special relationship with the UK Agricultural Research Council (ARC) as does Celltech with the Medical Research Council (MRC). Celltech was also set up by BTG (or, more precisely by the National Enterprise Board which has since fused with the National Research Development Corporation to form BTG) and operates in the field of biotechnology. Its relationship with MRC allows Celltech first refusal on any discoveries within the realms of recombinant DNA and cell hybridization that emerge from the various units of MRC. In return Celltech channels

"I'M SORRY, THE SCIENTIFIC DIRECTOR IS IN CONFERENCE."



money back into MRC and has an obligation to help the council find other commercial outlets for discoveries in which it has no interest.

ARC and many of its employers who work on subjects that will be of interest to the new company (some of whom are already consultants to BTG) tend to be enthusiastic about the prospect for several reasons.

The first is the possibility of an easy and financially beneficial way for ARC to channel its discoveries of commercial potential in the domain of plant breeding. At present it has no such outlet. New strains of plants developed by conventional techniques within ARC institutes have to be given to the National Seed Development Organisation which, although a profitable concern, donates its profits to the Treasury rather than directly to ARC.

For discoveries in gene cloning and transfer, that have some potential for being used in the genetic improvement of plant

strains, ARC has no ready outlet but individuals have sometimes had to contend with the rather persistent advances of some of the several American companies that have been set up in advance of any British counterpart. A special relationship with a company, preferably British, would therefore suit ARC well.

For its part, BTG is believed to have board approval for starting the company and to have a managing director in mind. It has not, however, yet found a scientific director and has met with some refusals already.

One problem facing any potential scientific director is the need to ensure that the company is profitable within five years. That was also the BTG stipulation for Celltech but the problem is greater in plant breeding because the genetic manipulation of plants by modern techniques is far less advanced than is that of bacteria, which Celltech use to make products of commercial value, such as rennin.

Nevertheless the optimists believe that profits could be made within five years by concentrating initial efforts on the improvement of bacterial strains, particularly the nitrogen-fixing *Rhizobium*, which are used to inoculate crop plants and on the development of techniques of clonal and meristem propagation.

Finance for the new company is being arranged by BTG which is almost certain to provide at least one third of the money from its own, governmental coffers. The rest will be raised from private sources.

Peter Newmark

University admissions

Still squeezed

Applications from home and European Community students for undergraduate courses at British universities are likely to be six per cent up this year on last, according to figures released by the Universities Central Council on Admissions (UCCA) (see table). But admissions to courses starting in October 1982 are expected to be down on admissions in October 1981.

This is no surprise. The government has implemented its policy of limiting university places just when the number of 18 year olds in Britain is increasing. Hence, the previous policy of gearing university places to demand has been abandoned. The Department of Education and Science, in recent evidence to the House of Commons Public Accounts Committee, has thrown

light on how this policy may affect potential students. The table below shows the department's estimate of the numbers of potential students that may be deprived of a university place in the academic years 1981-82 to 1983-84.

Understandably, universities hope to make up the fees lost from home students by taking in more students from overseas. But their aim has been made more difficult since the government removed the subsidy from overseas student fees. The figures clearly show that full economic fees have deterred a high percentage of potential overseas applicants.

The number of overseas admissions, however, is not necessarily a constant proportion of applications. UCCA statistics suggest that the shortfall in acceptances of places from overseas students in October 1981 over October 1980 was only 19 per cent, compared with the 34.5 per cent shortfall in applications. But statistics compiled by the University Grants

Annual percentage change in applications for undergraduate courses at UK universities

	1980 over 1979	1981 over 1980	1982 over 1981
Home students	+3.5	+4	+6*
Overseas students	-12	-34.5	-20*

*Estimates.

Committee (UGC) suggested an even smaller shortfall in overseas admissions: only two per cent in 1981 over 1980. This apparent discrepancy seems to be explained by the fact that UGC includes admissions for more non-degree undergraduate courses than does UCCA. So it seems that overseas students are now opting for shorter, less costly courses.

Last week, the House of Commons Public Accounts Committee published the report of its findings on the administration of university grants. The committee seemed pleased with the move by the education department to reduce the amount of university income not subject to cash limits by transferring the grant paid to home students for their fees directly into the universities' purse. The universities are praised for keeping their student intake on target last October (a 4 per cent shortfall over the previous year) — but the polytechnics, and other institutions of higher education, come in for a drubbing for increasing their intake by 18.2 per cent over the previous year. The committee's report urges that the body now being set up to control higher education outside the university sector be developed quickly with full co-operation of UGC and laments the fact that steps to coordinate all aspects of

higher education had not been taken before the cuts to the universities.

The parliamentary committee also seemed satisfied by assurances that the universities are taking care not to offer new tenured appointments with no redundancy clause. The Committee of Vice-Chancellors and Principals has been looking at more flexible forms of contract.

Judy Redfearn

US research support

Question of size

Washington

In coming weeks, the US Congress will probably pass legislation that would give a major shot in the arm to small research and development firms in the United States, many of which suffer in the present economic climate, yet which are major sources of new technical inventions. The legislation is not final, however, and is subject to considerable opposition voiced by spokesmen for the universities and government-sponsored basic research.

The National Science Foundation (NSF) estimates that there are 13,000 small firms in the nation, defined as independently owned firms with 500 or fewer employees and performing research and development work. Although numerous, accounting for 85 per cent of firms involved in such work, these small companies in fact spend only 4 per cent of all industry research and development dollars. In contrast, giants such as McDonnell Douglas and IBM spend 87 per cent of US industry's investment research.

Yet there is ample evidence that most innovations come from small firms. One 1976 study showed that small firms produce 24 times as many innovations per research and development dollar as large ones, even though the small firms receive only 2 per cent of total federal support for industrial research.

Small business found a champion last year in Warren Rudman a freshman republican senator from New Hampshire. Rudman introduced a bill that would set aside one per cent of all federal research and development funds — which total some \$40,000 million — for small firms. They would compete for the money by applying to separate federal agencies for grants, awarded on a peer review basis, as seed money. If the work was fruitful, some firms would qualify for follow-on funding. In a third phase, the money would have to come from the private sector, or from other government sources if the government was interested in the company's work.

The plan was modelled on the Small Business Innovation Research Program run by NSF, and a newer, similar programme run by the Department of Defense. The NSF programme gave some \$5 million in seed money to 42 small firms in 1977. By 1981, the 11 of them that qualified for follow-on funding had

No. of home students, aged under 21, entering university (thousands)

	1980-81*	1981-82	1982-83	1983-84
UGC targets	67.3	65.2	63.1	60.9
Target to maintain 1980-81 age participation rate†	67.3	68.5	70.4	69.5

* Actual intake

† Age participation rate is the percentage of 18-21 year olds in the population entering university, which was 7.5% in 1980-81.

succeeded in raising \$41.4 million in outside capital and equity. Moreover, the programme apparently created jobs. The 11 firms had employed 261 people in 1977; by 1982 they employed 616 people. The most spectacular growth was in a genetic engineering firm, Collaborative Research of Lexington, Massachusetts, which received \$25,000 in seed money in 1977 and by 1982 had raised \$24.9 million from outside sources.

The Rudman bill made a spectacular passage through the Senate in December. Of the Senate's 100 members, 85 were co-sponsors of the bill, and it passed by a vote of 90 to 0. One modification exempted the \$10,000 million in-house federal research and development from the calculation. A second modification was an amendment introduced by Senator Harrison Schmitt limiting the amount of funds to be set aside that could be taken from federal basic research budgets. This amendment was an attempt to placate spokesmen for the basic research community and universities who attacked the bill as a raid on basic research funds. They argued that development work in most federal agencies has powerful protectors, whereas basic research does not. In the Department of Defense, for example, the contractors and armed services buying the MX missile, or Trident submarine, would keep their research and development funds from the amount set aside, so that the basic research funded by the Department of Defense would be unduly thinned.

One fear being raised by university spokesmen is that the small firms' share of the federal research and development pie will grow, at the universities' expense. The proposed one per cent sounds modest enough, but any amount would take some funds away from federal basic research at a time when such money is becoming scarce.

Some university spokesmen argue that small firms do not do basic research of high enough quality to qualify for federal funds, and that a set-side programme will allow them to adhere to this lower standard. They argue that such firms should compete with universities and other traditional research groups. Several federal agencies prohibit for-profit firms from applying for research grants, although the National Institutes of Health has now lowered this barrier.

In the coming weeks the House will have to decide which version of the legislation it will pass. The variant most palatable to university spokesmen is that proposed by Don Fuqua, chairman of the House Science and Technology Committee. This would leave oversight of the programme to the authorizing committees of Congress for each of the federal agencies involved, thus allowing them to devise individual set-aside programmes or exempt the agencies under their jurisdiction.

The version most likely to pass, however, is a bill put forward by John J. LaFalce, which is modelled on the original

Rudman bill but is even friendlier to small business. The LaFalce version would make the money set aside not one, but three per cent of all federal research and development, and does not exempt federal in-house research from the calculation. The LaFalce version would make \$1,200 million available to the small firms in the first year — contrasting with the more cautious Rudman bill, which phases in the programme, reaching the \$300 million level in the third year. But in view of the opposition to the set-asides that has surfaced elsewhere in the House, it seems likely that the LaFalce forces would be satisfied with a final version limiting the set-aside to one per cent, having a three-year phase in period, and a feature limiting the "raid" on basic research.

Deborah Shapley

Polish Academy of Sciences Slow progress

Poland's new legislation on the Academy of Sciences will ensure parity of funding for the institutes of the academy and the research institutes of the production ministries, according to Warsaw radio. A main concern of Polish scientists has been the lack of separate budgets for the various institutes funded on the principle of dividing research into "problems" funded nationally. The new bill, which is under discussion by the Council of Ministers (Cabinet), thus seeks to redress one of the major grievances of the academy scientists expressed at last September's National Congress of Solidarity in Gdansk. It therefore forms part of a current tacit policy on the part of the ruling Military Council for National Salvation (WRON) to grant various "social" demands from the Solidarity programme while keeping open the question of the future of the independent trade union movement.

Much, however, remains uncertain, and nothing has been announced so far about one of the most contentious issues — the status of the Secretary of the academy. At present, the incumbent of this post holds quasi-ministerial rank, and is responsible in the first instance to the prime minister, not to his fellow academicians. During "renewal", as part of the nationwide drive towards "self-governance", there were strenuous moves (headed by the academy lobby within Solidarity) to change this anomalous state of affairs and ensure greater autonomy for the academy, thus ending the long-standing friction between the members and scientific employees of the academy on the one hand, and the academy bureaucrats on the other.

There has also been no news since the military council took power of many other proposed reforms, despite their apparent innocuousness. For example, it was proposed that the academy should decide, on purely academic grounds, whether or

Call from arms

Washington

At its annual meeting in Washington the National Academy of Sciences (NAS) made one of its rare ventures into public policy pronouncements. The assembled members adopted a resolution calling on the President and Congress and their counterparts in the Soviet Union "to intensify efforts to achieve an equitable and verifiable agreement" limiting strategic arms, and to "reduce significantly the number of nuclear weapons and delivery systems". The resolution further urged them to reduce the risk of accidental war, to inhibit proliferation of nuclear weapons, and to "continue and observe" all arms control agreements including Salt II, signed by the United States and the Soviet Union in 1979 but not ratified by Senate. Finally NAS urges the avoidance of "military doctrines that treat nuclear explosives as ordinary weapons of war".

The NAS resolution makes no mention of the "nuclear freeze" urged by other groups around the country. It was passed almost unanimously, with a few abstentions and one dissenting vote. Proposer for the resolution was Marvin Goldberger, president of California Institute of Technology and chairman of the academy's Committee on International Security and Arms Control. The resolution was sent to the President via his science adviser, George A. Keyworth II.

Deborah Shapley

not its members should be able to travel abroad. At present, non-scientific criteria still play a major role in such decisions. The emergency regulations on foreign travel for scientists stress that the would-be traveller must be given a thorough political vetting.

Not surprisingly, this can pose problems for academy scientists. A case in point is that of Artur Swiergiel, a young physiologist who, since last October, has been researching at the Babraham Institute of Animal Physiology in Cambridge.

Mr Swiergiel had a six-month scholarship under an agreement between the British Council and the Polish Academy of Sciences. Last November, realizing that he would need extra time for his experiments, Mr Swiergiel applied for an extension. On 31 March, he received a telegram from Professor Maciej Zurkowski, director of the academy's Institute of Animal Breeding and Genetics, confirming the extension. Three weeks later, a second telegram arrived, stating that Professor Zurkowski had been informed by "the academy" that the extension had been refused. No explanation was given — but Mr Swiergiel had formerly served on the Warsaw regional executive of Solidarity.

Vera Rich

Muscular dystrophy research

Positive move

Not content with the paradoxical increase in public donations to charities in times of recession, the Muscular Dystrophy Group of Great Britain (MDG) is aiming to raise an additional income of £1.5 million from industry over the next four years. And to coordinate the use of this money, earmarked for supporting clinical trials and work on the X-chromosome with that of their regular income, MDG have created the full-time post of research development director and filled it with an eminent scientist.

At the age of 59, Professor Arthur J. Buller takes on the new job. In his career Professor Buller has moved across the spectrum of bodies supporting UK medical research, serving as a member of the Medical Research Council (MRC) from 1975 to 1981, becoming dean of the Faculty of Medicine at Bristol University in 1976 and moving to the government's Department of Health and Social Security as Chief Scientist in 1978. Now he feels less optimistic about public sector support for research and views the drain on university funds and the cutback in government support for research as a serious threat to the dual support system. Medical charities can, he feels, ameliorate this situation.

MDG works on an annual budget of £1.5–2 million raised through 450 voluntary bodies (the Muscular Dystrophy Association of the United States has a budget in the region of \$16 million). In 1981 a total of £1,321,719 was made avail-

able for research — 45 specific projects have been approved for the academic year which began in October 1981. The biggest handout will go to the group's own research laboratory in Newcastle (£189,254). Two London hospitals will receive over £100,000 for work investigating tissue culture in dystrophic muscle and in the development and differentiation of single muscle cells.

The additional money gathered by the fund-raising committee will be diverted to what the group describes as "significant new research developments". It is hoped that £1 million will go to work on the X-chromosome. The techniques of recombinant DNA and gene cloning are already being applied to the X and other chromosomes with the dual aims of developing reliable methods of prenatal diagnosis and discovering the underlying genetic defect of several incurable diseases. Such work is already funded by the MRC and the Cystic Fibrosis Trust but MDG want to make certain that efforts are specifically directed to Duchenne muscular dystrophy, a disease linked to an unknown defect of the X-chromosome.

The other £0.5 million that MDG hope to raise from industry will be spent on clinical trials. The assessment of these is likely to be helped by the availability in a London hospital of a £200,000 nuclear magnetic resonance machine. This was purchased with the assistance of MDG and will enable muscle metabolism to be studied without taking tissue samples.

MDG is a working example of how medical charities can give invaluable support to research when public sources

are running dry. It may also prove that charity can keep new research initiatives at home — additional funds help stem the brain drain.

Jane Wynn

Ecology in the Sinai

Time of change

The final hand-over of the Sinai last week brought to an end an era of strict conservation and ecological surveying. Although there had been proposals, at the time of the Camp David talks, that the Sinai should become an "International Ecological Park for Peace", these have failed to materialize. Moreover, according to Israeli ecologists, Egyptian plans to develop the Sinai could lead to the repetition of Israeli ecological mistakes committed with the best intentions by the early settlers.

Under the Israelis, three major ecological centres had been established: a bird migration monitoring station at El Arish on the Mediterranean, a field studies school near the Santa Caterina Monastery, and the Ras Muhammed underwater observatory. None of these is still in operation, although a special agreement was signed ensuring the continuation of the Santa Caterina school. The unique coral habitat at Rad Muhammed has been virtually destroyed by soldiers fishing with hand-grenades.

Under Israeli control, ecological protection in the Sinai reached an almost ludicrous level, with tourists forbidden to pick up seaweed or coral from the beach for fear they might then drop it in the desert and upset the ecological balance.

The Egyptians have ambitions plans for developing the Sinai, but Israeli ecologists know all too well the ecological hazards of the pioneering spirit — citing drainage of the Huleh swamps in North Galilee, and Ben Gurion's plans for intensive development of the Negev.

Whether or not in cooperation with Israel, the Egyptians are matching Israeli developments. They are particularly interested in developing solar ponds as energy sources, and Israeli plans to replenish the Dead Sea from the Mediterranean. One Egyptian plan envisages an artificial "Dead Sea" in the Quattana depression, south-west of El Alamein. As this lies below sea level, the canal bringing in Mediterranean water could be used to generate power, while the resulting salt lake could, as in Israel, be concentrated by evaporation to form the basis of a chemical industry.

The emotional and political overtones of the return of the Sinai to Egypt make it unlikely that the Egyptians would seek Israeli advice on how best to develop the area. However, the Israeli ecologists, who have worked long hours during the past few months to make their Sinai studies as complete as possible, express themselves willing, to make their data available to the Egyptians.

Vera Rich

Musical chairs at the CEA

The French Commissariat à l'Energie Atomique (CEA) is to be reorganized, little more than a decade after the reforms of André Giraud set the CEA on its feet again.

In Giraud's time, the problem was that the CEA had lost a major battle over the choice of power reactor for a French nuclear programme: CEA wanted the indigenous French gas-cooled system, whereas Electricité de France backed the American pressurized water reactor (PWR). Electricité de France won, and the broken CEA had to be given new confidence. Giraud achieved that, and also helped set up France's nuclear industry, now so successful, behind the PWR.

Why the need to reform again? In part, CEA has outgrown the Giraud structure. It now controls many subsidiary companies whose management needs to be rationalized. But, more importantly, the new broom of the socialist government is to sweep clean. The catchwords are decentralization and regionalism. "Some reform of the CEA would have had to take place anyway" a CEA spokesman said last week "but it's going

to go further with the new government than it would have done with Giscard d'Estaing".

M. Michel Pecqueur, CEA's administrator-general, is to announce details of the reforms this week, with the hope — if the trade unions agree — that the budget for next year should be determined under the new structure. That means things must move fast, for the budget process begins in June.

One key issue will be the role of the seven *délégués*, who control the key sectors of CEA (nuclear applications, reprocessing, research and so on). Some say that these Paris-based posts carry too much power, and there must be some devolution to the units that the *délégués* control — among them the fundamental research laboratories at Fontenay-aux-Roses. However, one of the *délégués* last week expected their roles to remain much the same after the reform. The names of the posts might be changed, but the duties would not. Indeed, it is claimed that the reforms will not be revolutionary. No doubt that is CEA's intention.

Robert Walgate

MRC research unit

Job vacancy

The immediate future of the British Medical Research Council's (MRC) Pneumoconiosis Unit in South Wales seems assured, although life there remains unsettled. Early last year, the unit had been the subject of a regular review by the MRC which had recommended staff cuts and greater emphasis on research supported by industrial contracts. The fears of staff that the MRC was preparing to wind down the unit for final closure in 1987 when the director was due to retire, were heightened when the director, Dr Peter Elmes, left his post last January. Those fears were allayed, however, when the MRC announced that it would appoint a new director — advertisements will be placed shortly. In the meantime a four-person committee is in charge of the unit's daily administration.

Dr Elmes's departure, however, highlights a problem for a handful of MRC units whose work is largely vocational. Those units must win some of their money from contracts placed by the Health and Safety Executive, to whom the MRC still hands over some of its budget under the Rothschild customer/contractor principle. Frustrations can arise when the contracts

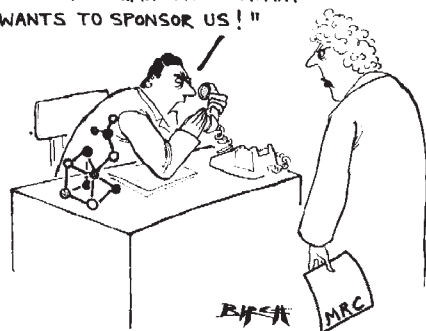
industry has increased. Dr Elmes, however, was not happy about the changes. He mentions delays to research work caused by lengthy negotiations with industrial customers, both at the outset of a piece of research and during its progress, and industry's reluctance to publish results quickly.

An illustration of some of the difficulties is provided by the case of kaolin workers in Cornwall. The MRC, according to Dr Elmes, had been unwilling to support studies into the causes of an excess of lung disease in the workers on the grounds that they did not constitute fundamental research. Industry and the Health and Safety Executive, which did come up with the money, awarded funds piecemeal for individual studies as the project progressed, thereby causing delay. Publication of the final results, which apparently suggest a link between kaolin exposure and lung disease has been delayed because of industry's desire to reduce the risk before it is widely known.

The MRC believes that many of these problems can be avoided if the terms under which research is conducted are made clear to industry at the outset. Hence, the council would like the Pneumoconiosis Unit to continue its efforts to win more industrial contracts. But the precise balance between applied and fundamental research, it says, remains to be seen. One important factor in determining the unit's structure will be the views of the new director.

Judy Redfearn

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are for routine toxicological studies which divert staff from fundamental research for which the units are primarily intended.

The MRC has used the problem to argue for an adequate budget to maintain fundamental research. But it has also urged the units to win more contracts from industry as well as the Health and Safety Executive to help it out of its fiscal difficulties. The more general problem, of course, is that routine toxicological studies on occupational hazards must be done and there are few suitable independent laboratories outside the MRC.

The MRC's plans for the Pneumoconiosis Unit have begun to take shape over the past two or three years. Hence there has been a shift in emphasis from clinical to *in vitro* research and the unit's staff has been reduced. Since the MRC's review early last year, the unit has received regular visits from a committee to advise on its work and research commissioned by

US science education

Nearing a crisis

Washington

Mr George A. Keyworth II, the President's science adviser, termed the "next few years" as "critical in science education in the United States". But he has extinguished any hopes that the Administration will do anything about it until the next presidential term.

In a speech to the National Science Teachers Association (a group whose ability to carry out its job has been impaired by Administration cuts of all funds for pre-college science programmes run by the National Science Foundation (NSF)), Keyworth repeated the unfortunate truths many US leaders have learned of late about how poorly, and how little, science and mathematics are taught in US schools.

For example, these subjects consume only seven per cent of elementary school teaching time, and even less time at the secondary level. The ability of students in science and mathematics has deteriorated steadily, and most people leaving school and going into non-technical professions have little idea of what science and technology are about. The public, he noted, spends \$100,000 million annually to run US public elementary and secondary

schools, while it spends around \$6,000 million a year playing video games.

Keyworth absolved the federal government of any role in solving the problem, except to produce graduate students in science. "The alarm being raised about reductions in federal support for science education is misplaced. We ought to be alarmed that those organizations and people spending the really big amounts of money seem to assign low priority to science education.

"Our most important job is to work together to convince our local communities, school boards and universities that science is as basic as history, that students who must study English in the twelfth grade must also study maths. But the federal government cannot and will not make this happen. We parents, teachers and citizens must take up this challenge directly."

Rebutting this argument is F. James Rutherford, who designed some of the high school science programme set up as a response to the Soviet Union's Sputnik, and who testified a week after Keyworth's speech to the opposite effect. He said that US elementary and secondary education is so decentralized and so locally controlled that no one is spending "really big amounts of money". There is no central focus for change or curriculum development except at the federal level, said Rutherford, who is now chief education officer of the American Association for the Advancement of Science.

Keyworth had argued that the post-Sputnik science education effort simply did not take root and that geography and demography worked against it. Rutherford says the decline was a direct result of earlier cuts in the NSF programme for science education (which once consumed half of NSF's entire budget). The Reagan Administration, for two years, has run down the remaining \$80 million of that money so that only \$15 million will be left in 1983 and this is allocated to graduate student fellowships.

Rutherford notes that the timing of the Administration's initiatives precludes anything further happening during Reagan's current term as president. The Secretary of Education, Terrel H. Bell, has established a National Commission on Excellence in Education, due to report in October 1983, when the government's 1984 budget will be virtually complete. John Slaughter, director of NSF, has said that his most important task as NSF director will be to set up another commission, on pre-college education in maths, science and technology, to report in late 1983. Thus these studies would have no impact, Rutherford notes, before the 1985 budget at the earliest, after the next election.

So while Keyworth calls the problem critical, he does not propose to do anything about it, except to have the teachers lobby their communities, and weep.

Deborah Shapley

CORRESPONDENCE

Sequence libraries

SIR — Thank you for your article "Europe leads on sequences" (*Nature* 15 April, p.596) on our efforts to establish a nucleotide sequence library. I welcome the attention given to the problems of data collection — a general discussion of these matters is both timely and important.

I do feel, however, that a few points require clarification. First, credit for the library at the European Molecular Biology Laboratory must also be given Kurt Stueber (of the Genetics Institute, Cologne), from whose collection we started, and who continues to work closely with us. Similarly, any discussion of nucleic acid sequence data collections in Europe must mention Professor Richard Grantham's group at Centre d'Evolution Moléculaire (Lyon), who have run a large data bank for many years.

Although a central data library has yet to be established in the United States (later this year according to NIH), several groups there have maintained large and excellent data bases for some time. The largest of these are the collections of Dr Walter Goad and his group at Los Alamos Scientific Laboratory, and Dr Margaret Dayhoff and her group at the National Biomedical Research Foundation (Georgetown).

This is not a case of "too many data banks". The different approaches taken have each generated their share of good ideas, and if the present cooperative atmosphere can be maintained, a really excellent international resource combining the best ideas can result.

GREG HAMM

European Molecular Biology Laboratory,
Heidelberg, FRG

"Useless" research?

SIR — *Nature's* leading articles show a persistent concern for the well-being of British universities, lately concentrating on research support and on the dual support system, at the expense of more important matters.

We are all concerned to protect research but I believe you seriously underestimate the resilience of the British research effort and its supporters. The latter are strong, vocal advocates of their cause and I know of no good reason why we should be alarmed at this moment. Much of the fundamental research in question is expensive and in no way urgent. It will survive a little slowing down and a little less international competition.

Of much greater concern is the industrial-economic basis of the country and the contribution to that basis which needs to be made by universities and, of course, by other agencies of higher education. In this context you mention (without, however, developing) the need for educational diversity. It is an essential requirement of any successful organization that it possess the highest degree of diversity compatible with its integrity. The model for a successful university system should be the usual model of any evolving system, namely a dynamic steady state stabilized in this case by the inevitable boundary conditions of budgets, standards and student numbers. It is essential that, within the system, there be both lateral and longitudinal flexibility in subject content and student effort. Such a pattern leads inevitably to the co-existence of broad degrees and

specialized degrees, of vocational and non-vocational options, of universities and polytechnics, and of low level and high level exit qualifications. There needs to be a marked change in the emphasis of university teaching towards the Design-and-Make Society and I would remind you of the Royal Society of Arts initiative in support of Education for Capability.

There is nothing radically wrong with British universities that a little loosening up will not cure. The present financial crisis, for example, will be coped with readily if all those over 60 or, if necessary, over 55 will simply make way for the young and for any subsequent changes in subject emphasis deemed necessary. Making way does not imply "walking the plank", but rather continuing one's vocation in teaching, research or both in retirement or semi-retirement. Some mortgaging of our financial future might still be necessary but not for long. Not every university can be saved an ugly confrontation in this way but the system as a whole could be. Since I am in favour of the present number of universities, a little more even spreading of the costs and the cuts would be entirely in order. Any suggestion that this would deprive us of necessary excellence is nonsense.

In winning back a little of the country's confidence in us, it would be as well not to go on supposing that our present arrangements are the best of all possibilities. A glance at France, Germany and the United States would convince many that this is not so.

The single honours degree is not the be-all-end-all. For its own good, the dominating influence of Oxbridge and its concentration on scholarly excellence and other platonic virtues will have to be challenged by other equally exacting paths of excellence, paths of university study which also lead to competence and usefulness. If it is true that the annual toast of the Cambridge Philosophical Society is or was "Here's to our researches, may they always be useless!", is it surprising that our industries falter? This simplified analysis is no doubt open to criticism, but I shall be content if it stimulates discussion of ways forward rather than of defences of the *status quo*.

GRAHAM HILLS

University of Strathclyde Vice-Chancellor,
University of Strathclyde,
Glasgow, UK

Research accountant

SIR — In an otherwise admirable article on current affairs and research at the Imperial Cancer Research Fund in *Nature* of 15 April 1982 (p.595), Robert Walgate is misleading in his interpretation of the accounts.

The income for the year ended 30 September 1981 is correctly stated to be £17 million, but £12.8 million (75 per cent) was spent on running the laboratories and extramural units, and £803,389 (4.7 per cent) on appeals, whilst a further £2.7 million (15.8 per cent) was spent on supporting the Liverpool cyclotron and trials with interferon. In addition we earmarked a sum of £2.5 million towards the cost of replacing the research laboratories that we shall lose when the Burtonhole Lane, Mill Hill premises revert to the MRC in 1986.

A.B.L. CLARKE

Imperial Cancer Research Fund,
London WC2, UK

Not all cranks

SIR — In his review of Gardner's *Science: Good, Bad and Bogus* (*Nature* 28 January, p.351) Sir Peter Medawar asks "was it not Cuvier who named a fossil ichthyosaur as *Homo diluvii testis* — man-witness-of-the-flood?"

No, it was not. And to make matters worse it was actually the good baron who showed the beast for what it was: a Miocene salamander. Later in his review, Medawar states "Not all scientific nonsense is written by cranks though: quite a lot of it is the work of scientists who . . . asseverate upon difficult subjects of which they have no deep understanding". Perhaps together with Medawar's "electrician-eugenicists" and "astronomer-microbiologists" we should include the immunologist-palaeontologist? Perhaps this is too harsh for I have no intention to Shock (*ley*) — it is just that Medawar has given little quarter to the many, doubtless deserving, persons who have come under his fire in the past and I only wish to see him play his own game according to Hoyle.

ANDREW FORESTER

Institute for Environmental Studies,
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April Fool!

SIR — In your April Fool's Day issue, the article on pages 392–393 ends with the statement, "Long life is a fishy business indeed". There is more than long life that is "fishy" here. You have been caught in a pseudonym web again, although this time indirectly. The authors of the article from *Acta Gerontol. (Prag.)* are kidding somebody, as "Dlouhy-Zivot" means long life in Czech, and "Ryba" means fish. Anyway, hyphenated names in Czech are rare.

FRANK A. PITEKKA

University of California,
Berkeley, USA

Creative energy

SIR — As a priest with a keen interest in astronomy and cosmology I have long pondered over the apparent contradiction to the second law of thermodynamics that is present in the standard big bang theory. I therefore read the paper "A model for the cosmic creation of nuclear energy"¹ with much interest.

Having attempted to exorcize the notion of a literal interpretation to the opening chapters of *Genesis*, I was amused to read in the above article, "We find that the main creation of nuclear energy started around 10 s after the big bang, and most of the exergy was created during the first few minutes, 85 per cent during the first hour, and that the process was essentially completed during the first 24 h." Perhaps we also wish to add, "And there was evening and there was morning, one day."!

"The first day of creation, who can act rationally on such a day!" — Alexander Solzhenitsyn.

GARTH BARBER

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1. Eriksson, K.E. Islam, S. & Skagerstam, B.S. *Nature* 296, 540–541 (1982).

Institutional biosafety committees and public participation: assessing an experiment

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THE regulation of recombinant DNA research has evolved at a pace almost as dizzying as that of the research itself. Only seven years ago, the scientific community voluntarily halted certain experiments while potential hazards were investigated. In 1976, the National Institutes of Health (NIH) issued regulatory guidelines mandating specific methods of physical and biological containment, and prohibiting high-risk experiments. Since then, regulatory controls have been steadily relaxed in light of evidence that risks may be less than initially feared. But some experiments are still considered potentially hazardous, and disagreement persists about whether the research poses long-term or low level risks¹⁻³.

Another major trend has been regulatory decentralization. In 1978, revised guidelines shifted primary authority for enforcement from NIH to locally-appointed institutional biosafety committees in order to simplify administrative procedures and to encourage local responsibility, although NIH continued to monitor committee decisions⁴.

Effects of NIH guidelines

Since 1978, the authority of biosafety committees has expanded to the point where virtually no federal oversight remains. Greater discretion has also been delegated to individual researchers; less than 15 per cent of permitted experiments now require prior approval from the biosafety committee. Retrospective review enables the committees to monitor safety standards without impeding most experiments. A recent report by the Congressional Office of Technology Assessment calls the guidelines "a comprehensive, flexible, and non-burdensome way of dealing with the physical risks associated with recombinant DNA research while permitting the work to go forward"¹.

The 1978 guidelines also instituted significant changes in public participation in decision-making. NIH's Recombinant DNA Advisory Committee was broadened to include more individuals from fields outside the biomedical sciences, each biosafety committee was required to include at least two members not affiliated

with the institution (often called "public" members) to represent community interests. Further, biosafety committees were required to make the minutes of their meetings available to the public upon request and were "encouraged" to hold public meetings whenever possible.

These provisions were adopted in response to charges by public interest groups and others that decision-making had been dominated by scientists and technical experts^{5,6}. They evoked sharply

The most innovative aspect of institutional biosafety committees, responsible in the United States for local oversight of recombinant DNA research, is mandatory participation from outside the institution. A survey of Californian committees and selected national data reveals wide variability in committee structure and procedures. Public participation, although constrained in various ways, has been generally constructive.

divergent reactions. Many scientists were openly sceptical about the public's involvement in complex technical issues^{7,8}. Some warned — recalling Lysenkoism — that it could lead to political repression⁹. Others saw a greater danger in the widening rift between science and society, and looked to increased public involvement in science to heal this rift¹⁰. The 1978 guidelines clearly struck a compromise between the basic changes proposed by critics¹¹ and the pleas of scientists to "quietly dismantle the whole hateful (regulatory) artifice" (ref. 7). Yet, these provisions did offer the possibility of a direct public voice in decisions at the local level and, in this sense, launched an experiment in public participation in science policy.

This study assesses the success of that experiment based on a survey of biosafety committees in California and national data. We focus especially on public participation because this is the most innovative aspect of the regulatory system, as well as the most controversial. The findings suggest that lay members have played a constructive role on biosafety committees — although constrained in various ways — and that involvement has been generally worthwhile.

The time is ripe for such an assessment. In November, the Recombinant DNA Advisory Committee considered making all the NIH guidelines voluntary rather than mandatory, but instead left them mandatory while again reducing their scope and placing still more responsibility for monitoring on biosafety committees. (This recommendation was recently adopted by NIH.) Despite this expanding

role, biosafety committees have never been systematically evaluated. Previous plans announced by NIH for a two-year national study met strenuous opposition from biosafety committee chairpersons¹² and have not been pursued.

The regulation of recombinant DNA research symbolizes, to many, the way that society will deal with scientific and technological innovations involving potential risks. For such issues, as Court of Appeals Judge David Bazelon has noted,

the only measure of confidence possible may be in the process by which decisions are made¹³. Thus, extraordinary effort has gone into developing a regulatory framework for recombinant DNA research that could provide sound, legitimate decisions responsive to local concerns; the Office of Technology report calls this framework "a possible model for societal decision-making on technological risks". The evidence presented in this paper points to certain strengths and weaknesses, but should not substitute for a more thorough investigation.

Survey methods

Data on Californian biosafety committees were obtained from surveys of chairpersons and nonaffiliated committee members. Questionnaires were sent to each chairperson of the twenty Californian committees registered in June 1980 with the Office of Recombinant DNA Activities of NIH. Nineteen committees responded, including twelve in academic institutions, five in non-profit research institutes and two in private corporations. Questionnaires were also sent to all of the 48 nonaffiliated committee members asking about committee performance and their own roles; forty-five questionnaires were returned, a response rate of 94 per cent. Due to these small sample sizes, few differences are statistically significant. Those that are significant are noted.

National data are based on a transcript of the plenary session of a meeting of about 200 biosafety committee chairpersons and other representatives, held in November 1980¹²; and on a brief survey completed by

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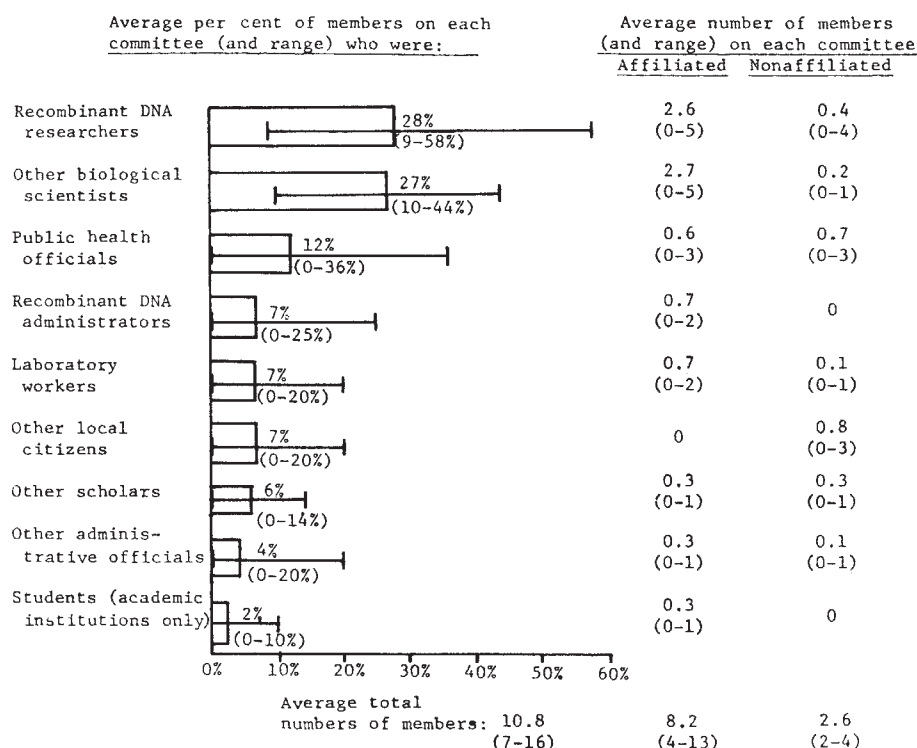


Fig. 1 Membership of California biosafety committees.

98 of the 100 participants in the meeting's health surveillance workshop (survey results provided by S. Barban, Office of Recombinant DNA Activities, NIH).

Survey findings

Since biosafety committees have had considerable discretion in interpreting guideline requirements, we describe first some general patterns of operation before discussing results on public participation.

Most striking is the great diversity in almost every aspect of committee activities from the frequency and content of meetings to the performance of mandated functions. For example, five of the Californian committees met once every three months during 1979, while several did not meet at all and one met fifteen times. Most of these meetings were convened as the need arose; in fact, eight of the nineteen committees reported no regularly scheduled meetings. About a third of the committees conducted some business by telephone or letter, several functioning this way almost entirely. Some committees delegated substantial authority to chairpersons; others relied heavily on subcommittees. Some committees were concerned only with recombinant DNA while others considered other potential biohazards.

The number of research proposals reviewed also varied greatly. Several California committees did not review any research proposals in 1979, while one reviewed 68. In both the Californian and national samples, about 60 per cent of all biosafety committees reviewed ten or fewer

proposals. Overall, committees spent about 30 minutes per proposal in meetings, but averages ranged from less than 10 minutes in one committee to more than an hour and a half in another. In general, smaller committees reviewed fewer proposals and spent more time on each of them. Some committees spent most of their time reviewing proposals while others concentrated largely on policy issues and other business. In California, committees at private corporations devoted the least time to policy and committee business (20 per cent), while those in academic institutions devoted the most time to such matters (57 per cent).

The guidelines require that institutions ensure "appropriate training" for biosafety committee members, principal investigators and laboratory staff; conduct "appropriate" health surveillance of recombinant DNA research personnel; and adopt emergency plans for accidental spills¹⁴. But since specific criteria are not provided, committees have interpreted these responsibilities quite differently. Nationally, the majority of committees offer no formal training courses. Training for committee members in California varied from none to a combination of university courses, manuals and laboratory tours. In the national sample, 89 per cent of the committees delegated responsibility for training laboratory staff to principal investigators, although several also assigned partial responsibility to the biological safety officer or to the biosafety committee.

Plans for emergency spills also differed considerably. Some Californian

committees tailored plans specifically to recombinant DNA research, others relied on standard safety procedures. In the national survey, 13 per cent of the respondents reported that their biosafety committees had no emergency plans for serious accidents or contamination, and 51 per cent reported no such plans for work-related illnesses. Although serious accidents in recombinant DNA laboratories were thought to be rare, more than a third of the respondents said that their committees would probably not know if any had occurred. About a third also said that laboratory staff were not told they were at increased risk of infection if pregnant or taking certain drugs. Overall, 15 per cent of the respondents reported that their committees did not do a "good job" in protecting the health and safety of laboratory personnel.

The majority of institutions had no special health surveillance for recombinant DNA laboratories. In California, 13 of the 19 committees reported no such surveillance, for reasons ranging from "lack of evidence of medical hazards" to the statement that "routine monitoring procedures are adequate". Likewise, fewer than half of the committees in the national survey had "established medical programmes". Most industrial biosafety committees reported that employees were given standard comprehensive medical examinations modified only slightly for recombinant DNA laboratory personnel. Participants differed as to what form local surveillance should take (routine physical examinations and serological sampling were generally not seen as useful), but they did agree that the present uncoordinated approach would not allow detection of low level health effects.

Approval of research projects

The Californian biosafety committees approved 96 per cent of research proposals reviewed in 1979, 73 per cent of them without modification. Only 4 per cent of all proposals were rejected, and the majority of committees (11 of 19) rejected none of the proposals considered.

There was again variation among committees. One, however, required modification of every proposal reviewed, while four approved all proposals without change. Rejection rates varied within a narrower range (0-10 per cent). The two corporate committees approved the largest proportion of proposals as submitted, compared with those in academic and research institutions (86 per cent versus 76 and 61 per cent); required the fewest modifications (14 versus 19 and 36 per cent); and had the lowest rejection rates (0 versus 5 and 3 per cent).

High approval rates could mean that only high quality proposals were submitted, that proposals had already been modified through informal consultation, or that the committee's review was not

sufficiently critical. From the data to hand we cannot say which interpretation is most valid, although informal consultation appears to be common. Most local decisions were eventually confirmed by NIH; since 1978 the Office of Recombinant DNA Activities has rejected only an estimated 5 per cent of proposals approved by biosafety committees (statement of W. Gartland, Office of Recombinant DNA Activities, NIH, at 25 September 1980 meeting of the Recombinant DNA Advisory Committee).

Once proposals are approved, a number of committees apparently take no further formal responsibility for enforcing the guidelines. At the national meeting, about a third of the committees represented in one workshop did not monitor laboratory procedures after initial protocol approval. Yet, as the workshop leader pointed out, problems are more likely after equipment has been operating for a while. Because continuous monitoring is important, the guidelines include periodic review of experiments among the committees' mandated functions¹⁵. Evidently this requirement has been widely ignored.

Composition of the committees

The guidelines leave the choice of committee members to the institution, but require each committee to include at least two non-affiliated "public" members, and to have a "biological safety officer" if research requiring P3 or P4 containment is being conducted¹⁶. In California and elsewhere, most members are appointed by the administration. In Cambridge and Amherst, Massachusetts, the city governments are also involved in selection, but such external involvement is unusual. Most committees comply with the membership requirements, although the national survey revealed several institutions that had P3 facilities and no biological safety officer.

The Californian committees ranged in size from 7 to 16 members, averaging 11, and all had the required number of nonaffiliated members. Figure 1 shows the average composition of these committees, and reveals wide variation. For example, recombinant DNA researchers comprised anywhere from 9 to 58 per cent of each committee, and there was comparable variability for most membership categories. The "typical" committee consisted of 55 per cent recombinant DNA and other biological scientists, with five to ten per cent of the membership in each of the other seven categories. Scientists were in the majority on most committees; only two contained fewer than 50 per cent scientists and one had more than 90 per cent. Six of the 19 committees were composed entirely of men, and all were chaired by men. Otherwise, there were few consistent patterns.

The guidelines state that the non-affiliated members "shall represent the

interest of the surrounding community with respect to health and protection of the environment", and they list as suitable examples "officials of State or local public health or environmental protection agencies, members of other local government bodies, or persons active in medical, occupational health, or environmental concerns in the community"¹⁷. Many of the Californian committees followed these suggestions: the two largest categories of nonaffiliated members were public health or other government officials (33 per cent) and local citizens (31 per cent).

The third major category reflects a rather different response: 25 per cent of all nonaffiliated members were recombinant DNA or other biological scientists working at different institutions. In two committees, the non-affiliated members consisted entirely of outside scientists. Although most such scientists were well qualified technically, their qualifications for representing community interests on public health or environmental issues were generally not evident from their *curricula vitae*.

A major premise underlying the proposals to broaden participation in biosafety committees was that local citizens and other non-scientists would raise different issues from scientists, offering contrasting perspectives. Data from the Californian survey support this premise. Predictably, lay nonaffiliated members had more trouble understanding discussions than did scientists (66 versus 43 per cent), and tended to rate their own contributions as less valuable. Several lay members reported that their suggestions were given little weight. One commented that "the committee served mainly as a rubber stamp . . . The tasks require specialized expertise . . .".

On the other hand, lack of technical understanding could sometimes be advantageous. As one lay member said, it "requires the committee to think at a less hurried pace about potential problems . . . to think through problems from a somewhat different perspective", and to seek "information that a trained person might have assumed was implicit". One said simply, "I am the gadfly."

Scientists and non-scientists also had different views about community influence. Lay nonaffiliated members were a good deal more sceptical than nonaffiliated scientists about direct

community input to committee proceedings. Yet the nonaffiliated members, apparently because they viewed themselves as representatives of the community, were almost twice as likely as the scientists to say that committee decisions "always" took account of community views (38 versus 20 per cent).

Value of public participation

Committee representatives at the national meeting disagreed about the value of lay members. Some thought that such members contributed little. Others claimed that community members played an important role — that they helped to avoid potential conflicts of interest and "caused the committee to operate in a more tidy fashion". Several stressed the useful political function of public members in providing a sense of open communication with the community and in defusing potential animosity.

Do the benefits of public participation come at the cost of increased inefficiency or incompetent review? The Californian survey provides no evidence of such a tradeoff. Committees with local citizens reviewed almost twice as many research proposals in 1979 as those with no citizen members (20.4 versus 10.9 proposals), at the same time spending a somewhat larger fraction of meetings policy issues (36 versus 26 per cent) and covering more such issues (2.8 versus 2.5 per cent). Furthermore, although committees with citizen members spent less meeting time per proposal (27 versus 37 minutes), their review does not seem to have been more superficial than that of other committees, as they had a slightly higher rate of proposals rejected (6 versus 1 per cent) and of modifications requested (24 versus 21 per cent). These differences were independent of committee size, even though committees with citizen members tended to be larger, and larger committees tended to have lower approval rates. Within both smaller and larger committees (Table 1), those with citizen members had lower approval rates as well as higher rates of modification and rejection.

With lay members in the clear minority on most committees, it is perhaps surprising to find even small differences. These findings are consistent with psychological research indicating that lay people tend to define technical issues more broadly than experts and to be more

Table 1 Comparison of rates of approval of research proposals for institutional biosafety committees with and without citizen members, by committee size

	Committee size			
	7-10 members		11-16 members	
	No	1-3	No	1-3
	local	local	local	local
	citizens	citizens	citizens	citizens
Research proposal	70	63	98	81
Approval rates				
Per cent approved as is	29	35	2	7
Per cent approved with minor modification	1	3	0	11
Per cent rejected				
Number of biosafety committees	5	7	3	4

cautious in assessing possible risks¹⁸. And they bear out James Watson's warning that "public members . . . may take regulation seriously, unlike the molecular biologists"⁷.

Public access

Community representation can also occur by direct participation in committee meetings. Thus, the guidelines "encourage" open meetings whenever possible¹⁹. Many commentators had urged stronger measures — mandatory open meetings that were publicized and held at convenient times — fearing that a voluntary provision would have little impact.

Such fears were justified. The Californian survey as well as the national meeting indicate that most committees have done little to encourage public participation; many have actively discouraged it. Almost half (42 per cent) of the Californian committees held no open meetings. Of the eleven committees that did have "public" meetings, only five held them on a regular schedule, in all cases on a weekday during working hours. More significantly, these meetings were apparently not announced. When queried about different forms of publicity, not one committee reported any announcement of meetings on bulletin boards, in campus or local newspapers or in other public media. At the national meeting, most chairpersons also reported that their committees did not publicize meetings widely. It is hardly surprising that public attendance has been minimal. About half of the Californian committees holding "public" meetings reported that no one attended while others had typical audiences of one or two.

As in 1978, many still dispute the value of open meetings. Almost half of the committee chairmen in California felt that open meetings were not desirable. Objections raised included the use of meetings as a "political" forum, possible violations of confidentiality, inhibition of frank discussions by committee members and fear that a lay audience ignorant of

technical issues would impede the committee's operation.

In committees that had open meetings, however, almost all the chairmen thought they were desirable, for reasons ranging from preventing public misconceptions to informing committee members about community views. The performance of these committees compared favourably with those with closed meetings with regard to number of proposals reviewed and policy issues discussed (Table 2). And, like committees with public members, those with open meetings spent slightly less meeting time per proposal, yet were somewhat more critical in their judgements, rejecting more proposals as well as requiring more modifications. These differences were independent of both committee size and citizen representation. They suggest that open meetings do not make committees measurably less efficient and may, in fact, lead to a more critical review of proposals.

Effects of open meetings

If there are differences between open and closed meetings, they probably have less to do with specific issues raised by audience members than with subtle changes in atmosphere. Most people attending committee meetings asked general questions about research procedures or policies such as earthquake standards or laboratory safety. Yet comments from nonaffiliated members suggest that open meetings may nonetheless have had an effect on the committee. As one member noted, "community members are the only 'outsiders' at the meeting", adding, "Without them, who are the watchdogs?"

Assuming that open meetings facilitate direct communication with the community, one would expect committees with open meetings to receive more views from the community and to be more responsive. Table 2 shows just the opposite pattern. Nonaffiliated members on committees with open meetings more often said that the community had no input and that committee decisions did not always

adequately account for community views. One explanation may be that, as one member put it, open meetings "sensitize academic members to community concerns", leading to greater awareness of the ways in which the committee was not responding to the concerns they heard. Alternatively, members of committees with open meetings may simply have had higher expectations concerning community influence. Despite scepticism about the amount of community participation, however, almost all nonaffiliated members rated it as somewhat or very helpful.

Minutes of meetings provide another source of public information about committee proceedings. The guidelines require that such minutes and related documents be made available upon request²⁰. Here again, this requirement has had a negligible impact; only one of the Californian committees reported receiving requests for minutes. Militating against such requests was the fact that the meetings were unannounced, and the minutes were typically stored in an administrative office not readily accessible.

There is a potential conflict between public accountability and the privacy necessary to protect trade secrets, as the guidelines acknowledge. So far, such conflicts have arisen mainly in proprietary institutions. At the national meeting, corporate committees reported that meetings were not open to the general public. Outside members, many paid by the companies, had to guarantee confidentiality, further limiting public accountability. In California, only the two corporate committees reported restricting the agendas of meetings because of proprietary concerns, although four other committees took special precautions to protect potentially patentable results before releasing minutes.

Are biosafety committees worthwhile?

Most chairpersons at the national meeting seemed to think that the time and effort spent by biosafety committees were greatly out of proportion to the risks of recombinant DNA research and therefore that the committees were largely unnecessary. Opinions expressed in the Californian survey were strikingly different. More than 80 per cent of the chairmen thought the guideline requirements concerning biosafety committees were "about right", while 91 per cent of the nonaffiliated members agreed that "biosafety committees as they presently function are worthwhile". This difference in views may be due not only to changes in perceptions of risk between 1979 and 1980 but also to the different sources of data — individual questionnaires versus the group process that produces a consensus.

Comments in the California survey, as in the national meeting, highlighted functions

Table 2 Selected comparisons of biosafety committees with open and closed meetings

	Committees that have:	
	No open meetings	50-100% open meetings
Average number of research proposals reviewed in 1979	14.1	18.0
Average meeting time spent per proposal (minutes)	56	51
Proposal approval rates:		
approved as is	79%	70%
approved with modification	20%	24%
rejected	1%	6%
Average number of policy and procedural issues discussed that were listed by chair	1.6	3.5
Per cent of nonaffiliated members who said community had no input into committee proceedings	38%	58%
Per cent of nonaffiliated members who said committee decisions did not always account adequately for community views*	47	78
Number of biosafety committees	8	11
Total number of nonaffiliated members	18	27

**P* < 0.05.

of biosafety committees. Many nonaffiliated members believed that the committees had an important role in promoting safety; for instance, one commented that they "force researchers to meet strict standards, as enforced by the campus environmental health officer. Several hazardous projects have either been aborted or redesigned because of our action". Others thought that biosafety committees provided a useful forum for internal review of controversial issues and also "a sense of security to the public agency and to the scientific community — no secrets, open communication".

Discussion

In combining technical evaluation of scientific issues with provisions for accommodating social values and limiting conflicts of interest, institutional biosafety committees represent a significant policy innovation. The experience of the Californian committees — which appear to be typical of committees elsewhere — suggests three general conclusions about their performance.

First, the considerable diversity of biosafety committees appears to reflect not only varying local circumstances but also variable effectiveness. Furthermore, the varying approaches (if any) to surveillance prevent development of a standardized system that might be more capable of establishing safety or detecting hazards.

Compliance has been variable even on specific requirements of the guidelines. Not all institutions conducting P3 research have appointed biosafety officers, and many do not monitor experiments after initial approval. Continuous monitoring is also hindered by infrequent committee meetings. The Office of Technology Assessment reports that biosafety committees "usually meet monthly", but only one of the 19 California committees met that often in 1979, and the frequency of meetings has undoubtedly declined since then. Stanford's committee, which met four times during 1979, now meets only once a year unless special problems arise (interview with D. Perkins, chairman of the Stanford Biosafety Committee by M. London).

The low rates of rejection of research proposals also raise questions about effectiveness. While these low rates may result from high quality proposals, they may also be due in some cases to inadequate review by the committee. Previous studies of similar institutional committees monitoring human subjects research, suggested that their low rate of rejection of proposed experiments was at least partly due to poor performance.

A second conclusion that may be drawn is that, given the highly decentralized nature of the biosafety committee system, clear minimum standards for essential elements of the system would be helpful. One of the key experimental variables in

these committees has been public participation, yet the guidelines define this variable only very generally through the requirement for nonaffiliated members. The stated intent is that such members shall represent the surrounding community with respect to health and the environment; and the regulations stipulate that institutions are expected "to adhere to the purpose of the guidelines as well as to their specifics"²¹. But precisely *how* community interests are to be represented is left notably ambiguous, inviting disparate interpretations. Thus, a quarter of all nonaffiliated members were biological scientists from other institutions, many engaged in recombinant DNA work and with no evident qualifications for representing community interests.

More specific provisions concerning public representation, such as those recently adopted by the Food and Drug Administration for human subjects committees²², could have avoided this ambiguity. Provisions had been proposed in 1978 that committees include a certain proportion of non-scientists or reflect the demographic composition of the community, to ensure direct community involvement (following the examples in the guidelines) rather than representation by outside scientists¹¹. But NIH rejected these proposals on the grounds that the biosafety committee "is in large part an expert committee whose essential function is to evaluate research protocols in respect to containment levels, using the explicit instructions of the guidelines. Rigid quotas are not necessary"¹⁴. With such ambivalence about the role of public members, it is not surprising that the regulations remained ambiguous.

It would also have been useful to define what constitutes "appropriate" training for biosafety committee members. Insufficient technical instruction compounded the frequent difficulty that many nonscientists had in understanding discussions, and a number stressed the need for more systematic training on basic research techniques and terminology.

The role of the public

Minimum standards for critical factors such as technical support and provisions for community representation need not restrict local experimentation in meeting or exceeding these baselines. Such standards are vital in a decentralized regulatory system where effectiveness depends largely on local structures and procedures. Without mechanisms for assuring accountability to community interests, public participation in biosafety committees has not been fully tested.

Yet it is clear that some committees did make a genuine effort to involve local citizens and were at least partially successful. The results of these experiences suggest a third conclusion which, although tentative, is perhaps the most important:

given a chance, public participation seemed to work fairly well. Most community representatives took their role seriously and contributed to effective committee performance by broadening discussion and encouraging more critical scrutiny of research proposals. Public meetings, even with limited attendance also appeared to give committees a better understanding of community concerns without impeding committee operation. Many members believed that the committees were an important channel for public communication about an issue that remains sensitive, and that public involvement caused no evident harm to the research or to science.

Even if federal regulations are eliminated, the controversy over how to regulate recombinant DNA research is not likely to disappear. It has recently emerged anew in communities where genetic engineering companies have been set up. Because NIH regulations do not cover commercially-sponsored research, several towns and states have recently passed or drafted ordinances extending the present guidelines to industrial research and adding other requirements¹⁸. Thus, biosafety committees or their equivalent may well continue to be a key element in local regulation. As a workable mechanism for direct public participation, these committees have established a significant precedent in the decision process concerning science and technology.

We thank Drs William Gartland, Stanley Barban and Elizabeth Milewski of the Office of Recombinant DNA Research Activities, NIH, for making records available, Nancy Pfund and Ralph Silber for helping design and conduct the California survey, and Natalie Fisher, Valerie Herman and Kathy McFadden for technical assistance. Funding was provided under a grant from the NSF's EVIST Program and the National Endowment for the Humanities.

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NEWS AND VIEWS

New frequency standards from ultra-narrow Raman resonances?

from Peter Knight

THE high-resolution study of atomic and molecular resonances over the past decade has led to the establishment of an atomic time standard based on the very stable atomic beam microwave transition between the hyperfine ground states of the caesium atom. Laser excitation of atomic or molecular transitions offers potential improvements in this 'standards' field but has been dogged by linewidth problems. The lasers jitter in frequency and the optical transitions investigated have been broadened by motion, collisions or by spontaneous decay, so that the gain in frequency over the microwave transition is offset by the increase in linewidth. Much effort is being expended to produce more stable lasers and to generate narrower spectral lines, in part because of a general spectroscopic interest and in part to find new laser-driven atomic clocks that could supplement or even supplant the Cs clock. This could meet new demands for ultra-precise time intervals. A recent experiment reports a new ultra-high-resolution laser spectroscopic excitation which not only eliminates laser jitter problems but also almost all line-broadening mechanisms, including that of a finite lifetime, and leads to a spectral line whose centre is stable to great precision.

Ezekiel and his colleagues at MIT and the Rome Air Development Center have measured an optical atomic resonance of width 650 Hz (Thomas *et al.* *Phys. Rev. Lett.* **48**, 867; 1982). This width is four orders of magnitude less than the inherent natural width of one of the states involved and almost certainly less than the linewidths of the phase-jittering lasers used to excite the transition. This remarkable example of a 'subnatural' linewidth has major implications for frequency and atomic clock standards. Already, the preliminary data are claimed to compare well with the short-term stability of a conventional Cs atomic beam clock. Ezekiel's

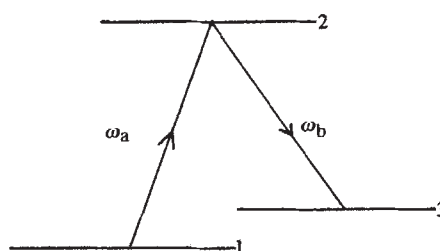


Fig.1 Resonance Raman transitions.

group use stimulated resonant Raman transitions (Fig.1) with transitions from states 1 to 2 and 2 to 3 driven by lasers of frequency ω_a and ω_b .

Conventional atomic beam clocks drive directly the hyperfine transition from state 1 to 3 in Cs with a microwave radiation field. A single microwave cavity of length l interacts with atoms of velocity v for a time $t \sim l/v$ as they fly through in a beam, and will excite a resonance at frequency $\omega \sim \omega_{31} \equiv (E_3 - E_1)/\hbar$ of width of order $\Delta\omega = t^{-1} \sim v/l$ by simple Fourier arguments. States 1 and 3 are both ground states and are not broadened by any other influence (there is no Doppler broadening in the microwave-beam interaction, there are no collisions in the dilute beam and no natural broadening of the line). To decrease this transit-time width $\Delta\omega$ and increase the resolution $\Delta\omega/\omega_{31}$ suggests increasing l . In fact, two separate microwave cavities separated by a distance $L \gg l$ and fed in phase are used. The atom has a probability amplitude a_1 of being excited in the first cavity and a_2 in the second. The transition probability $P = |a_1 + a_2|^2$ has a two-slit interference pattern due to the quantum interference between different routes from indistinguishable initial and indistinguishable final states. The interference lineshape is called a Ramsey resonance and has a width $\sim (v/L) \ll (v/l)$, making possible ultra-high-resolution spectroscopy.

An optical transition between the ground state 1 and excited state 2 is not often limited by this transit-time broadening. Instead the linewidth is produced by

grossly larger influences. The Doppler motion-induced spread in frequencies amounts to several GHz, which can be circumvented either using the new non-linear laser spectroscopic tricks, or by exciting a well collimated atomic beam perpendicularly with a well collimated tunable laser beam. Providing the atomic beam is not too dense, collisional widths can also be eliminated. The excited atom still interacts with its environment: it couples to the vacuum-quantized radiation field and consequently decays spontaneously with a lifetime typically of order 10^7 Hz. This width seems immutable, and the spectral structure in the natural width forever unresolvable. However this is not the case: there are several ways in which atomic coherence can be exploited to generate sub-natural linewidths, and I have reviewed these elsewhere (*Comments atom. molec. Phys.* **10**, 241; 1981). Nevertheless, we would not expect more than an order-of-magnitude improvement from even the most accomplished subnatural spectroscopist, simply because the penalty paid for the decrease in linewidth is exponential loss in signal strength.

Ezekiel *et al.* have used stimulated resonance Raman transitions from state 1 to 3 via state 2, by the absorption of a photon of frequency ω_a from one laser field and the stimulated emission of a photon of frequency ω_b into another laser field, such that $(\omega_a - \omega_b) \approx \omega_{31}$. A peculiarity of such transitions is that the Raman interference line has a width governed by the sum of the widths of states 1 and 3 only, with no real contribution from 2. Proof of this requires extensive analysis of the dynamics of the laser excitation and is true only for Raman difference frequencies $(\omega_a - \omega_b)$ close to ω_{31} ; otherwise 2 will strongly contribute to the transition dynamics. If the frequency ω_a is chosen to be exactly resonant with the ω_{21} transition frequency and ω_b is scanned through the ω_{23} resonance frequency and the decay fluorescence from state 2 measured, a Raman lineshape is generated in the fluorescence intensity versus ω_b . Its overall shape is a Lorentzian of natural

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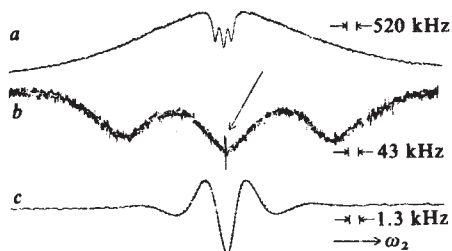


Fig. 2 Raman and Raman-Ramsey fringes observed by Ezekiel *et al.* in sodium (from *Phys. Rev. Lett.* **48**, 867; 1982). The arrow points to the fringes. See the text for details.

width (usually 10^7 Hz), but when $(\omega_a - \omega_b) \approx \omega_{31}$ (that is, at the 'two-photon' resonance frequency) a narrow non-absorbing 'hole' (G. Orriols *Nuovo Cim.* **B53**, 1; 1979) is burnt into the lineshape. At this hole frequency, the atom is entirely transparent. Physically the Raman (or two-photon) hole is due to atomic coherence. The atom is optically pumped by the two lasers into a coherent superposition of ground states 1 and 3 in which dipole moment induced on the 1-2 transition exactly cancels the 2-3 induced dipole. This vanishing total dipole moment leads to zero net absorption and consequently no decay fluorescence. The dipole moments only cancel close to Raman resonance: elsewhere population can be excited into 2 and contributes a fluorescent, naturally broadened line.

Ezekiel *et al.* chose to study resonance Raman transitions in a sodium atomic beam in which state 1 is the $3^2S_{1/2}$ ($F=1$) hyperfine ground state, 3 is the $3^2S_{1/2}$ ($F=2$) hyperfine state and state 2 is the $3^2P_{1/2}$ ($F=2$) hyperfine sublevel, of lifetime 16 ns. The atoms are excited by co-propagating laser fields, and Raman dips are observed on a 10^7 Hz-wide fluorescent background when $\omega_a = \omega_{21}$ and ω_b is scanned through ω_{23} . Three Raman dips are observed corresponding to Raman transitions between different magnetic sublevels in states 1 and 3, separated by the 300 mG Zeeman field applied to the atoms. Each has a width governed principally by the transit time $t \sim 1/\nu$ in the region simultaneously irradiated by the two laser beams. The two laser fields need to be correlated in their phase fluctuations or the two-photon, or Raman coherence will be washed out. Ezekiel's group eliminate this phase fluctuation problem by deriving one frequency, ω_b , by acousto-optic modulation from a stabilized laser of frequency ω_a , such that phase-jitter in the two beams is precisely correlated. Since Raman coherence depends in part on the phase difference between the light fields, the correlated jitter leads to a non-fluctuating Raman phase factor and to a Raman dip which can be, in principle, narrower than the laser bandwidth.

As the Raman, or non-absorbing, dip is uninfluenced by the upper state decay lifetime τ , a Ramsey double-excitation technique can be used with a long delay

$T \gg \tau$ without loss in signal but generating ultra-narrow lines similar to those in the Cs atomic clock. The two correlated frequencies ω_a and ω_b are mixed and used to excite the sodium atomic beam in two regions separated by a distance L . Figure 2 shows the sequence of lines observed for $L \approx 15$ cm. In Fig. 2a, three Raman dips are observed on a 10^7 Hz-wide naturally broadened fluorescence line as ω_b is scanned through ω_{23} excited in a single interaction region. A Raman width of 50 KHz is attainable. Figure 2b shows the expanded scan of the Raman dips, now excited by two interaction regions separated by 15 cm, with central fringes corresponding to Ramsey interferences on the central Raman line (Ramsey fringes on the other dips are washed out by stray magnetic fields). Figure 2c shows the further expanded scan of these central Ramsey

fringes. Increasing the interaction separation L to 30 cm produces fringes of width 650 Hz (HWHM).

If these Ramsey-Raman techniques were applied to the Cs hyperfine Raman transition, a short-term stability $\Delta\omega/\omega \approx 2.5 \times 10^{-11}$ seems possible over 1 s, which approaches the stability of a 30 cm interaction-separation Cs atomic clock. The laser technique can be improved by decreasing laser-induced Stark shifts, optical pumping signal enhancement, laser-cooling of atoms to increase flight times and perhaps by applying it to ions in electromagnetic traps. The new technique has arrived just as metrologists all over the world are seriously investigating new possibilities to replace the ageing Cs microwave atomic clock, and will be scrutinized carefully as a potential time-keeper of great stability. $\square$

Enhancing elements for activation of eukaryotic promoters

from Moshe Yaniv

TINKERING with recombinant DNA systems for the expression of genes in eukaryotic cells has recently thrown up a new class of regulatory sequences. These are the 'enhancers', short viral sequences that can stimulate, by up to two orders of magnitude, the transcription of coding sequences from their own promoters. Even more remarkably, they do so whatever, within reason, their position or orientation with respect to the coding sequences.

Enhancers were first discovered as sequences near the early genes of simian virus 40 (SV40) and polyoma that are required for virus viability¹⁻⁴. Approximately 30 base pairs (bp) upstream of these genes is found the TATA sequence typical of eukaryotic genes transcribed by RNA polymerase II and which appears to determine the specific site of initiation of transcription. In SV40, between 113 and 257 bp upstream of the RNA start point is a directly repeated 72 bp sequence. Removal of one 72 bp sequence has little effect; however, if part of the second sequence is removed the genome is rendered non-viable because at least one of the repeats is required for transcription of SV40 early genes.

It is now clear that viral enhancers can also exert their effects on cloned nuclear genes. Schaffner's group has shown that the SV40 72 bp repeat can increase the expression of a cloned rabbit β -globin gene in HeLa cells by 200-fold. The globin gene was introduced into the HeLa cells by the calcium phosphate co-precipitation

technique and its expression was measured after 2-3 days, before the integration of this DNA into the cellular genome. The SV40 72 bp repeat has to be covalently joined to the gene (that is, it functions in *cis*), but surprisingly it works even if it is several kilobases upstream or downstream from the transcription initiation site, and its orientation with respect to the gene is unimportant⁵. Similarly, Chambon's group has shown that the activity of the chicken conalbumin or adenovirus major late promoters can be stimulated by the SV40 enhancer⁶.

These observations clarify and extend earlier suggestions that viral sequences may affect the activity of eukaryotic genes. Capecchi's finding that the transformation of TK⁻ (a thymidine kinase mutant) mouse cells by plasmids carrying herpes virus *tk* gene is more efficient when the plasmid also carries the SV40 genome was probably mistakenly interpreted in terms of more efficient integration⁷. The stimulation of expression of rabbit β -globin gene in HeLa cells by a 244 bp DNA fragment from polyoma virus⁸, of human β -globin and in certain cases of the herpes *tk* gene by the SV40 72 bp element^{4,9}, and of the chloramphenicol acetylase gene linked to chicken α -collagen promoter by SV40 sequences¹⁰ are probably other demonstrations of enhancers in action.

Enhancers do not seem to be limited to papovaviruses. 72 bp directly repeated sequences are also found in the long terminal repeats that flank integrated retroviruses (for example Moloney sarcoma virus). Although these sequences have no obvious sequence homology with

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those of SV40 they can replace them to produce viable hybrid molecules¹¹.

The presence of enhancers in retroviruses may explain some recent intriguing findings that appeared to be inconsistent with the promoter insertion model of viral carcinogenesis¹². According to this model, integrated retroviruses can provide active promoter sequences that increase the expression of cellular genes. By integrating upstream of the cellular homologues of viral oncogenes, and consequently increasing their expression, retroviruses lacking their own oncogene are thought to be able to transform cells. However, Payne *et al.* have shown recently that in bursal lymphoma cells, avian leukosis proviruses can be integrated upstream or downstream and in either transcription orientation relative to the cellular *myc* gene (*c-myc*) — the cellular homologue of the putative transforming gene (*v-myc*) of myelocytomatosis virus. Clearly these findings are inconsistent with a simple promoter insertion model; however they fit nicely with the known properties of enhancers.

An interesting possibility is that enhancer sequences may have a role in regulating cell differentiation. Polyoma viruses are normally unable to grow in early embryonal mouse cells, or in differentiated derivatives of these cells. Mutants of polyoma with the capacity to replicate in embryonal cells were found to have sequence rearrangements¹³⁻¹⁶ in the region of the virus shown to contain enhancing sequences, although not of the 72 bp repeat type⁸. These results suggest that enhancers may be involved in tissue- or cell type-specific control of gene expression.

The mechanism of action of enhancers is unknown but several possibilities may be envisaged. Prominent amongst these are that enhancers may act as chromatin organizers, RNA polymerase entry points, sites of attachment to the nuclear matrix or regulators of local DNA superhelicity. Of course these possibilities need not be independent or mutually exclusive. Some evidence that enhancers may regulate

chromatin structure comes from their presence in regions of papovavirus genomes not packaged into nucleosomes in cells¹⁷⁻¹⁹. This may lead to changes in the structure of chromatin propagated on both sides of the enhancer and thus permit efficient transcription of neighbouring genes. Alternatively the nucleosome-free region may act as a high-affinity site for binding RNA polymerase²⁰.

How enhancers really work, and whether they have a role in the normal control of cellular gene expression, are clearly exciting problems likely to be the subject of much future research. Already it seems that genomic DNA contains sequences homologous to viral enhancers²¹, and what may be an enhancer has been found upstream of the sea urchin H2A gene²². □

The great galactic centre mystery

from Guenter R. Riegler

WHEN γ -ray astronomers discovered last year that the positron-electron annihilation radiation from the galactic centre region varied on a time scale of six months¹, it became necessary to reconcile possible explanations for the phenomenon with data from the radio and IR bands. This provided the impetus for a recent workshop* that examined the central parsec of the galactic centre using data from all available spectral ranges.

Images of the central few parsec of the Galaxy can be readily obtained through two experimental 'windows': bremsstrahlung emissions of ionized gas near 6 cm wavelength, and thermal emission of heated dust near 10 μ m. Both maps show a non-uniform brightness distribution¹⁰ and their similarity implies that the ionized gas and the heated dust are distributed along an arc-like structure.

Observations of the galactic centre at radio wavelengths show a non-thermal compact radio source surrounded by ionized gas moving at high velocities. The compact source varies on time scales as short as one hour², but we are not yet able to form a consistent picture of the actual source size. Neither ordinary pulsars nor binary stellar radio sources match the spectral index and luminosity of the compact radio source.

Maps of line emission of atomic hydrogen, ionized hydrogen and molecular gas (primarily ammonia and formaldehyde) show a complex distribution at all scale sizes from a few parsec to a few kiloparsec. The large, non-circular motion of a large portion of the gas may suggest it is being expelled from the centre, while the large velocity dispersion in the central parsec suggests a massive collapsed object. New searches for H₂O masers in the galactic centre, reported at the workshop by Güsten (Max-Planck-Institut für Radioastronomie, Bonn), imply that star formation is not the source of the high thermal flux in the galactic centre region.

IR observations³ show that ionized gas

in the central few parsec of the galactic centre is concentrated in at least 14 small clouds. An ionizing radiation of $\sim 10^{50}$ photons per s is required but its origin is debatable; sources might be distributed among, or in, each discrete IR source, or a central source of radiation might ionize all clouds.

Aitken and co-workers (University College London) found that peaks in the interstellar grain temperature coincided with the positions of IR sources (as deduced from peaks in 10.5 and 12.5 μ m continuum radiation), and argue that this implies that internal sources of luminosity power the discrete sources. With colour temperatures derived from maps at 5, 10 and 56 μ m, Rieke and Lebofsky (University of Arizona) argue that each IR source is heated by a cluster of stars of spectral type earlier than I. Gatley (UK Infra-Red Telescope) showed that most of the central parsec is transparent at UV wavelengths, so that heat sources need not be embedded in the IR-emitting dust clouds. The ionizing radiation may therefore be produced by a distributed source which is merely centrally concentrated about the position of IRS 16 (commonly accepted as the IR counterpart of the non-thermal compact radio source in Sgr A West).

The main argument for the existence of a central massive object at the galactic centre comes from a study of the dynamics of ionized gas clouds. Lacy³ found that the motions of the gas clouds are best fit with a mass distribution which includes a central mass in addition to a distributed mass, each of $\sim 3 \times 10^6$ solar masses ($M_{\odot}$). The central mass could be a star cluster, for example, a highly evolved OB association as favoured by Lacy.

The conjecture that there might be a black hole of mass $1-5 \times 10^6 M_{\odot}$ at the galactic centre was made more than a decade ago by Lynden-Bell and Rees⁴. Arguments for and against its existence were given by several speakers at the workshop. Lacy (Caltech) pointed out that a model of spherical accretion of ordinary

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*A workshop on the galactic centre was held on 7-8 January 1982 at the California Institute of Technology. The proceedings will be available from the American Institute of Physics, New York, in June 1982.

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interstellar gas towards a massive black hole would be inconsistent with the observed Sgr A IR spectrum and the required ionizing flux. The accretion rate which is required to power all activity in the galactic centre region, $\sim 10^{-5} M_{\odot} \text{ yr}^{-1}$, may also be augmented by debris from stellar disruptions or from nearby collisions involving giants. Some authors, particularly Ozernoi and his collaborators⁵, have argued against the massive black hole hypothesis on the grounds that the rate of star capture would be incompatible with observations. Rees (University of Cambridge) reviewed these arguments and showed that it is nevertheless possible to construct models in which the luminous flare from each stellar remnant's impact with the black hole would not lead to excessive average luminosities.

Charge-coupled device images of the galactic centre at an effective wavelength of $0.9 \mu\text{m}$ came from three groups and all showed a pair of faint, very red sources within a few arc seconds of IRS 16 and the compact non-thermal radio source. The sources are not as highly reddened as had been expected and it was suggested that line emission from doubly ionized sulphur may contribute to the observed photon flux within the bandpass of the CCD/filter combinations.

The positron-electron annihilation line at 511 keV has been observed by at least five experimental groups over the last decade and is, in fact, the only non-solar γ -ray line that has been confirmed by multiple measurements. The line emission⁶ implied an annihilation rate of 10^{43} per s, and a luminosity of $10^{37} \text{ erg s}^{-1}$, compared with an observed luminosity at IR wavelengths of $10^{40} \text{ erg s}^{-1}$ (the bolometric luminosity of the source of ionizing radiation in the galactic centre must be still higher by roughly an order of magnitude³). Furthermore, the luminosity of the annihilation radiation exceeds the radio luminosity (1.35–31 cm) of the non-thermal source⁷, and is a few hundred times more intense than the X-ray luminosity, measured by the Einstein Observatory from the central few parsecs of the Galaxy⁸.

The first satellite measurements of the 511 keV line from the galactic centre in 1979–80 showed that the emission had decreased by a factor of roughly three over a time span of six months¹. Recent experiments by Leventhal (Bell Laboratories) and Paciesas (Goddard Space Flight Center) did not observe a positive signal for line emission from the galactic centre, consistent with the assumed further decrease in intensity. Jacobson (Jet Propulsion Laboratory) pointed out that the available data are statistically consistent with a model consisting of an extended, constant-intensity source and a point-like, variable source of 511 keV line emission.

If the variable positron emission comes from a point source at the galactic centre,

then some inferences can be made about the positron source as well as the positron annihilation region. As pointed out by Ramaty (Goddard Space Flight Center) and Lingenfelter (University of California, San Diego), the width of the 511 keV line and its temporal variation^{1,6} require that the annihilation region have a temperature, density, degree of ionization and size which are consistent with the peculiar warm clouds³ and the other compact IR sources⁷ observed in the central parsec of the Galaxy.

The nature of the positron source is also strongly constrained by the observed line emission variation: multiple, extended sources such as pulsars, supernovae, black holes, or cosmic-ray interactions with the interstellar medium are excluded. Blandford (Caltech) suggested that the positrons might be produced by an electromagnetic cascade process in the magnetosphere of a $\sim 10^6 M_{\odot}$ black hole. In this model, high-energy ($\gg m_e c^2$) photons and electrons interact with much cooler ambient photons and gas. Lingenfelter and Ramaty point out that interactions in which the photon, electron and ion energies are all comparable, and of the order of $\sim m_e c^2$, are much more efficient for electron-positron pair production. The necessary energy could be provided either in the vicinity of a very compact source, like a black hole of $< 10^2$

$M_{\odot}$ (to agree with arguments by Ozernoi that the tidal disruption of stars would otherwise be inconsistent with stellar luminosity in the vicinity of the galactic centre) or by interactions of high-energy beamed photons with one another.

Oort (Leiden Observatory) described the galactic gas motion of scale sizes of $\sim 1 \text{ pc}$, $\sim 300 \text{ pc}$ and $1\text{--}3 \text{ kpc}$. The non-circular motions and the tilted distributions of the molecular clouds in the two larger-size regimes have been ascribed to either eruptive activity from the nucleus, or large-scale streamings in a non-axisymmetric potential field, but a unified model is apparently not yet available. Recent studies by Lake (Bell Laboratories) of orbits in three-axial ellipsoids have shown the existence of 'anomalous' orbits in tilted planes which may explain at least part of the observed features on the $1\text{--}3 \text{ kpc}$ size scale. The molecular clouds within 300 pc are, however, more likely to originate from an eruption in the nucleus of the Galaxy. $\square$

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Are Sahelian droughts predictable?

from S.I. Rasool

WHILE the controversy over whether the Earth as a whole is cooling down or warming up continues to rage unabated, there are a few large-scale climatic phenomena with major economic and social repercussions that are becoming amenable to analysis. One is the Sahel drought problem. Others include the circumstances which lead to the onset of the Indian monsoon and the impact of Pacific sea-surface temperature anomalies on North American climate and the upwelling of cold water off South America, with its important effects on fisheries and global climate. Unlike these latter phenomena, however, the Sahel drought has not been the object of coordinated measurements and intensive research. The lack of activity is all the more conspicuous in light of the enormous increase in the amount of climatic data acquired over Sahel in the last ten years.

Landsats 1, 2 and 3 have now provided

about ten years of uninterrupted observations over Sahel which can be used to estimate temporal changes in the surface cover and in the albedo. The man-made increase of albedo, impeding convection, has been put forward as the principal factor triggering the drought. The energy balance over Sahel and its variation at different times in 1978 and 1979 has also been successfully measured with Meteosat I. Meteosat II has now become operational and will continue to provide information on cloud cover, and on ground albedo, surface temperatures and atmospheric water vapour over Africa and the Atlantic. NOAA meteorological satellites are also giving daily coverage of meteorological parameters over most of the Earth. But how can one get the people with this vast quantity of data talking to those who need the data?

COSPAR (International Committee on Space Research) is keen to see how observations from space can help resolve some of the fundamental issues in the earth sciences and decided to sponsor a workshop on the Sahel climate problem. The OECD's Club du Sahel, which is interested in the economic development of

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the Sahelian countries, hosted the workshop and it was held in Paris in January.

The Sahel drought of 1969–73 was not considered to be unique as there have been other long periods of severe drought, notably in the 1910s and 1940s. Detailed analysis of data from some 1,200 stations in Africa (S. Nicholson, Clark University) suggests that the Sahel drought is related to rainfall anomalies on a continental scale, extending from southern Africa to just north of the Sahara desert, which characteristically persist for as long as 10 to 15 years at a time. Among possible explanations for recurrent long-term climate anomalies are the displacement of the Inter-Tropical Convergence Zone (for yet to be discovered reasons) and changes in global east to west air circulation patterns caused, in part, by changing conditions over distant oceans (Indian, Atlantic and Pacific) leading to lack of moisture over the Sahel at the beginning of the rainy season.

The persistence of drought may in part be caused by biogeophysical feedback mechanisms involving long-term changes in the heat balance of the Sahel, partly driven by changes in surface properties (such as albedo and soil moisture). Modelling studies (P. Rowntree, British Meteorological Office) indicate that soil moisture in the Sahel may have a significant effect on the circulation of the atmosphere and on rainfall over West Africa. Analysis of Landsat data (M.F. Courel, IBM Scientific Center, Paris) indicates an increase in surface cover and a decrease in surface albedo over northern Senegal in the past eight years.

The amount of dust blown from the Sahara Desert over the Atlantic Ocean is enormous, ranging from 100 to 400 million tons a year, and making up as much as one-half of all the dust in the Earth's atmosphere. The dust may change the energy balance of the ocean-atmosphere system over the Atlantic considerably (T. Carlson, Pennsylvania State University) and affect the climate of the Sahel and possibly of the whole world, although the precise magnitude of the phenomenon is not yet known.

Scientists from COSPAR at the meeting recommended that intensive analysis be made of surface and radiosonde data from the Sahel and Central Africa for the past 15 years. Such data are available but have not yet been completely assembled and analysed. Coordinated and intercalibrated analysis of satellite data from pre-drought, maximal drought and post-drought periods would give a better understanding of the mechanisms behind the onset of the drought, and its persistence in 1969–73. Over the past 10 to 15 years, satellites have furnished a large body of observations of the Sahel, neighbouring regions of Africa and the Atlantic from which information on cloud cover, dust, surface temperature, surface albedo, heat balance and soil moisture could be extracted. The

COSPAR scientists also suggested that a field experiment in the Sahel, within a 200×200 km area, be carried out to measure the vertical and horizontal fluxes of moisture and heat. Such a field experiment, within the grid of a standard general circulation model, could be designed based on existing ground and satellite networks and would provide a critical test for the general circulation model.

The scientists attending the meeting urged COSPAR, in cooperation with the

World Climate Program and the Club du Sahel of the OECD, to create an *ad hoc* working group to speed up implementation of these recommendations. All the necessary ingredients seem to be there: an enigmatic problem of extreme economic and social importance, the existence of large amounts of unanalysed data both from ground and space, and good scientists apparently available to start working on the problem. Get the right data to the right people and we may actually begin to see what causes the droughts in Sahel. □

Meteoritics: evidence for chemical fractionation in the early Solar System

from Robert Hutchison

CHONDRITES, the most numerous of meteorites, are stony types which have not been melted since they formed some 4.55 Gyr ago. They are complex and contain not only minerals that formed at high temperatures, but also volatiles that seem unlikely to have condensed from a gas of solar composition unless the temperature was below 700K. The siting of these volatiles has a bearing on theories of the formation of the ordinary chondrites which, in turn, place important constraints on theories of planetary formation. There are three main theories, explained below, of which the first two have the most supporters.

(1) When the various materials accreted, different proportions of volatile-rich fraction were included. Different parts of chondritic parent-bodies were subsequently heated and metamorphosed to different degrees, but without loss of volatiles. An unequilibrated chondrite, such as Tieschitz, thus contained, on formation, a higher proportion of 'Holy Smoke' (ultrafine-grained, volatile-rich matrix) than a metamorphosed member of the same chemical group, such as Allegan¹.

(2) Each chemical group of the ordinary chondrites had, on accretion, a uniform composition. Heating and metamorphism occurred in conditions where volatiles were lost, the loss being greatest in the types subjected to the highest temperature².

(3) A theory favoured by a minority, including myself and some of my co-workers, is that ordinary chondrites accreted when hot ($1,070 \pm 100$ K). Volatiles were most readily introduced into the material nearest the surface, which cooled most rapidly^{3–5}. No post-accretionary heat source is required.

It is clear that the first and third hypotheses lead to a theory of

heterogeneous accretion of planetary bodies, while the second favours homogeneous accretion. Choice of the most plausible theory is obviously crucial to our understanding of the origin of the Earth and makes identification and separation of the volatile-rich fraction in chondrites of particular significance.

It is not surprising, then, that over the past two decades there have been many mineralogical and chemical investigations of chondrites aimed at deciphering their thermal and chemical histories in the hope that part of the story of planetary formation may be pieced together.

Somewhat contradictory conclusions were reached in three of the most recent contributions to this field. Rambaldi *et al.*⁶ argued that in ordinary chondrites, the volatiles tend to be concentrated in 'Holy Smoke', some of which may even be identifiable in chondrite types which equilibrated at high temperature. In contrast, Ashworth^{7,8} identified ultrafine-grained, 'non-clastic' matrix as a minor component in a few highly unequilibrated ordinary chondrites only. Finally, Christophe-Michel-Lévy⁹ suggested that equilibrated ordinary chondrites accreted over a range of temperatures extending up to 1,270K, whereas an unequilibrated H-group chondrite accreted below 620K; only in this last case was fine, volatile-rich dust incorporated.

Part of the problem of these conflicting interpretations lies in the strengths and weaknesses of the different techniques used. Rambaldi *et al.*⁶ took samples of six ordinary chondrites and used freeze-thaw and/or gentle disaggregation followed by sieving to produce various-size fractions down to $0.08 \mu\text{m}$. Instrumental neutron activation analysis was used to determine up to 24 elements in each size-fraction and further, as yet unpublished, information, has been derived from the electron microprobe and the scanning electron microscope (SEM).

Ashworth used the high-voltage electron

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microscope on ion beam-thinned samples to identify minerals and examine textures at the submicrometre level, but detailed work is of necessity limited to very small areas. Christophe-Michel-Lévy's SEM approach looked at textures and minerals, with semi-quantitative chemical information on the latter, in essentially untreated fragments of meteorite. Each of the three approaches provides useful information which cannot be obtained using the other techniques; the chemical data of Rambaldi *et al.* cannot yet be had from single, sub-micrometre-sized grains.

Rambaldi *et al.*⁶ found that the finest size-fraction of each meteorite — that richest in 'Holy Smoke' — was generally richest in moderately volatile and volatile elements such as K, Zn and Sb; these fractions tended also to be enriched in refractory elements such as W and the rare earths. This is true of the H5, equilibrated chondrite, Allegan, but, except for Zn, Ag and W, not true of the unequilibrated, H3 Tieschitz. And here lies the problem. Although the distribution of the 24 elements between the size-fractions may be explained in general terms, difficulties arise with individual meteorites.

For example, Co, Ni and Fe are enriched in the finest fraction of Tieschitz relative to the two coarser fractions. Rambaldi *et al.*⁶ suggest that this may be due to the presence of a Ni-rich magnetite. However, Christophe-Michel-Lévy⁹ found the finest-grained matrix to be mainly iron-rich olivine, and Ashworth⁸ states that this 'dark matrix' has "troilitic and metal-rich varieties". Neither author found magnetite in Tieschitz. It may be concluded, therefore, that Rambaldi *et al.*'s finest size-fraction probably contains a mixture rich in olivine, metal and troilite, but with other phases too. A further observation of Christophe-Michel-Lévy⁹ helps clarify the reason why the rare earths La and Sm are depleted in the finest size-fraction of Tieschitz. She noted that in this meteorite, phosphate is associated with Fe-Ni metal, occurring largely as inclusions within metal grains. Phosphate has also been found as tiny crystals in cracks in metal where it probably formed from P, expelled from metal, by reaction with O and Ca from neighbouring 'dark matrix' (A.W.R. Bevan and R. Hutchison, unpublished).

Phosphates concentrate rare earths, and, although the crystals are fine-grained, most (and most rare earths) probably would have been retained in metal during the gentle disaggregation carried out by Rambaldi *et al.*⁶.

So what of 'Holy Smoke'? Search for a carrier of volatiles in the ordinary chondrites essentially began in 1973. In that year, Anders' group discovered that some whole meteorite samples of the brecciated H-group chondrite, Supuhee, are enriched in Ti and Bi relative to CI chondrite¹⁰. For several reasons CI chondrites are taken to be our best available sample of unfractionated condensable Solar System material. Enrichment of the volatile metals Ti and Bi in bulk Supuhee was therefore interpreted as indicative that a fraction must exist which is enriched in these metals by some hundreds of times relative to CI. This is because Supuhee is composed mainly of high-temperature minerals and so the

volatile-rich fraction — termed 'mysterite' — cannot occupy more than a few per cent by volume. Anders' group concluded that 'mysterite' represents a rare substrate on which Ti and Bi condensed at a late stage in the cooling of a solar nebula.

'Mysterite' or 'Holy Smoke' must, however, be widely distributed among ordinary chondrites. Tieschitz, for example, contains more Bi and as much In and Ti as CI¹⁰. Dodd¹¹ and others have shown that this stone contains only 8 per cent by volume of material with a grain size less than 100 μm , and it has already been stated^{8,9} that in this fraction most grains are of high-temperature minerals. If water and carbon, with other volatiles, are concentrated in the remainder of the fine-grained matrix, then this can constitute no more than about one per cent of the whole meteorite by volume. It is the search for, and characterization of, this type of material that now looms large in meteorite research¹². □

The aging of the brain

from Patrick Rabbitt

CONFRONTING an immanent future in which one-fifth of the population of the developed countries will be aged over 65, delegates to a conference* on the aging brain need not lack large general questions to attack. It is a comment on the present underfinanced and fragmentary state of research on aging that most papers were disjunctive presentations by research teams primarily known for their expertise in other areas. None of these teams can yet afford to commit all their resources to the systematic study of the aging central nervous system (CNS). For this reason there could be little discussion between groups interested in psychology and behaviour, in neuroanatomy and physiology, in neuroendocrinology, in neurochemistry and pharmacology or in animal models of aging.

Several important general problems of the aging CNS were illustrated, rather than illuminated, by the context in which they were raised. D. J. Reis (New York) and his colleagues neatly pointed out the increasing variability in performance which occurs among aging animals. This can be due to different rates of cell loss, both between different animals and between different areas of the same brain. Alternatively, rates of loss may be constant across different brains and brain areas, but this loss may start from different initial levels of neuronal density. Or, of course, variability in performance may be due to interactions between both these factors. Reiss and his group found differences in

local brain weight in the caudate nucleus and the olfactory tubercle in BALB and CBA mouse strains. These differences correlated with differences in behaviours thought to be mediated by dopaminergic neurone systems in open fields and in habituation to exploration of objects in dark slots in a test box. The strains also show different responses to amphetamine. This raises the fascinating possibility that specific differences in particular parts of the brain related to the presence or absence of single genes, or groups of genes, provide a direct link between genes, brain anatomy, neurochemistry and behaviour. The implications for relative cell loss with aging seem secondary to this important news.

For functioning human beings the most intriguing question about the aging of the brain is whether, individually or universally, we suffer a steadily accelerating loss of ability after some brief peak in our early twenties or whether, more happily, we can look forward to a long plateau of optimal function followed by a mercifully sudden 'terminal drop' in performance just before death which makes it irrelevant whether we have our wits about us or not. L. F. Jarvik (University of California, Los Angeles), who has studied a particular group of people, including some monozygotic twins, since the late 1940s, is one of the few investigators in the world who has data bearing on this question. She discussed

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*The 10th Aharon Katzir-Katchalsky Conference on 'The Aging of the Brain' was held in Mantua, Italy on 26–29 March 1982.

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psychometric data on 35 of these people as their average age rose from 64 to 73 years and found, overall, no significant decline and, indeed, an apparent improvement on some sub-tests as age increased. From 73 to 84 years the picture was less encouraging and, when group mean scores were considered, all test scores declined.

This does not yet answer the question since group mean scores might decline because particular individuals within the group might become ill and approach death just before testing, and the proportion of people showing such terminal changes in performance and contributing to mean scores would be certain to increase as the group aged. Jarvik's analyses of her data bore out this possibility. Individual subjects showed little or no decline in performance through the seventies and into the eighties. Moreover, abrupt declines in performance in individuals were good statistical predictors of impending death. Equally interestingly, elderly peoples' self-rating of

their own health status was also a good predictor of their actual future survival — as good as, but (perhaps disappointingly!) no better than, their physicians' predictions.

All longitudinal studies are certain to be criticized because at the time when they yield their richest data the focus of scientific interest is bound to have changed. One cannot, alas, time travel 20 or 30 years to include cases or use tests which become crucial as the state of the art advances. However, investment in really adequate longitudinal studies need only be very modest. Such studies are expensive to initiate, but very cheap to run once they are begun. Jarvik's study illustrates, but does not resolve, the great simple questions about changes in human efficiency with advancing age. If we want to know more about our own personal futures, and about the futures of the societies we have to plan, no single investment can yield so much useful information for so modest an outlay. □

Engineering organic molecular layers

from C.W. Pitt and I.A. Shanks

SCIENTISTS and engineers are taking a renewed interest in thin films of organic materials deposited, molecular layer by molecular layer, by an unconventional process attributed to Irving Langmuir and Kathleen Blodgett and dating from the 1930s. Such films can exhibit a remarkable perfection of structure over large areas and as the successive monomolecular layers may be of the same or different materials, a kind of 'molecular engineering' is made possible.

Long hydrocarbon chain molecules, with a hydrophilic group at one end and a hydrophobic group at the other, can be made to form a continuous film by spreading them on a water surface. The nature of the groups at the ends of the molecules ensures that one tip of the molecule is immersed in the water while the other remains in the air. A movable barrier which penetrates the water surface may be used to compress the film into a condensed layer exactly one molecule thick in which the oriented molecules are held together by van der Waals' forces. The film may then be transferred from the water surface on to a solid substrate by dipping the substrate through the surface and then withdrawing it whilst continuing to compress the film. Repeating this process allows a multilayer film to be progressively built up.

The Langmuir-Blodgett (L-B) technique

undoubtedly emerged at a time long before the technology to exploit its unique capabilities was available. A recent three day symposium* made it clear that the situation has now very much changed. Recent work has highlighted materials that have attributes which can be readily exploited when they are deposited as L-B layers. Many of these material systems have been developed as modified molecular structures tailored specifically for this deposition technique.

Typical of the molecular structures reported were the diacetylenes — a monomer/polymer system incorporating a conjugated triple bond which may be induced to cross-link using an electron beam or by UV or visible radiation. Diacetylene films are being investigated as gate region insulators in indium phosphide field effect transistors and as layers in optical waveguiding devices. Double bond molecules, such as ω -ticosenic acid, have been deposited as very thin electron beam resists capable of defining lines less than 600 Å wide, while even the simple fatty acids have found application, in very pure form, in modifying the surface properties of semiconductors used in Schottky barrier diodes and photodiodes.

A radically new approach to modelling biological membranes is also offered by the L-B technique. The fluid bilayer of lipid

molecules may be simulated by a simpler structure fabricated from reconstituted purified natural membrane components and laid down by the L-B process. Protein complexes, such as bacteriorhodopsin, can be immobilized in such layers, simulating the natural system. Possible applications of these biocomposite films include filtering solutions with chemically selective elements incorporated in the L-B layer. Entirely synthetic membranes, such as polypeptide layers, have also been fabricated, although applications are less apparent at this stage.

A technique for retaining the interesting properties of non-amphiphilic molecules, while enabling L-B deposition, is clearly emerging; that of attaching a hydrocarbon chain and a polar group to an aromatic ring-type molecule. Tetraphenylporphyrins, anthracene, perylene and pyrene were all reported as molecules which have been treated in this manner. Applications include tunnelling layers and electro-fluorescent display panels.

In addition to using these films in device applications they are also of interest as investigative tools in the understanding of a wide range of other phenomena. Examples are the use of manganese stearate monolayers to explore existing theories of two-dimensional magnetism and of monolayers of fatty acids and their salts to probe the mechanisms of the sliding friction between surfaces. The similarity between L-B multilayers and liquid crystals has also been noted and papers were given on the deposition and properties of *n*-pentyl cyanoterphenyl and other liquid crystalline materials. □



100 years ago

RARELY has so distinguished and representative an assembly been seen in Westminster Abbey as that which met to pay the last honours to Mr. Darwin, on Wednesday last week. The Abbey indeed was crowded. The character of the long line of distinguished men who followed the honoured remains to the grave, may be seen from the list of pall-bearers: — The Duke of Devonshire, the Duke of Argyll, the Earl of Derby, Mr. J. Russell Lowell, the American Minister, Dr. W. Spottiswoode, P.R.S., Sir Joseph Hooker, Mr. A.R. Wallace, Prof. Huxley, Sir John Lubbock, and the Rev. Canon Farrar, Mr. Darwin has been buried close beside the grave of Sir John Herschel, and within two paces of that of Sir Isaac Newton. From *Nature* 26, 16, 4 May 1882.

*A three-day symposium of forty invited scientists and engineers was organized by the Rank Prize Funds to explore the science and optoelectronic applications of Langmuir-Blodgett layers. The participants included both junior researchers in the field and eight invited speakers from Europe, the US and the UK.

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REVIEW ARTICLE

Human body clocks and the timing of sleep

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The alternation of sleep and wakefulness in humans exhibits surprising regularities during long-term isolation from the usual time-of-day cues. The inferred domination of our consciousness and physiological states by internal clocks may soon have practical implications for medicine and psychiatry.

WE ALL fall asleep from time to time and no one seems to know why¹⁻⁴. While obliged to adhere to a daily schedule of work and rest, light and darkness, most people go to sleep and wake again once a day at predictable times. The regularity of this schedule is imposed ultimately by the rotation of the whole Earth, but it turns out that rhythmicity persists even in the absence of any imposed schedule⁵⁻¹¹. There are some intriguing regularities about the timing of sleep onset and the subsequent wake-up in these conditions of so-called 'temporal isolation'. They reveal the presence of an internal clock with natural period just a little longer than the Earth's rotation period. Aschoff and co-workers were the first to recognize the influence of this clock on the timing of sleep and in the daily rise and fall of body temperature^{5,6,11,12,13}. There may even be two timers: one is a circadian clock with spontaneous period 24–25 h dominating the rhythms of body temperature, plasma cortisol and rapid eye movement sleep; the other seems to have a longer period governing the ebb and flow of sleep and associated physiological rhythms such as growth hormone secretion¹⁴⁻²⁰.

Prolonged temporal isolation

Inferences about the number and variety of timers affecting our sleep habits are drawn from data similar to those of Fig. 1. Here we see the times of sleep (black) and waking (white) of an individual living without external time reference for 4 months in a cave^{21,22}. Figure 1 replots the original data in the TV-raster format now conventional: the time axis is broken up into consecutive equal intervals (in this case, of 24.3 h) stacked from top to bottom. The whole picture is then duplicated to the right to emphasize the continuity of time from the right edge of each 24.3-h interval to the left edge of the next.

The black bars would be stacked in a vertical column if sleep and waking recurred at exact 24.3-h intervals. They do not. The intervals intermittently grow longer, shearing the stack of black bars to the right as it descends. The raster plot in Fig. 1 conspicuously depicts a slow but relentless change of organization in the timing of sleepiness. Czeisler²³ was the first to notice this in a diversity of such records. It appears that briefer records can often be assigned a place in a similar progression: sleep onsets tend to recur at longer and longer intervals, while still preferentially occurring near the temperature minima (recurring at 25-h intervals). Near the bottom of Fig. 1, sleep and waking alternate about once in every two cycles of 24.3 h. This phenomenon has been observed in humans at least since the cave experiments of Chauvet *et al.*^{21,22}. Isolated subjects, emerging from a month's studious or meditative confinement, exhibit understandable perplexity, even disbelief, that the time has passed twice as fast as they reckoned. During these 2:1

intervals, one subject on average slept 19 h at a stretch and stayed awake 31 hours before retiring again, never aware that his days were abnormally long. Such individuals typically eat somewhat more at three meals between rising and retiring, but not enough more to prevent weight loss (E. D. Weitzman, personal communication). Needless to say, this was at first alarming to observers outside, responsible for the subjects' well-being (ref. 11, p. 59).

Whatever the reason for this gradual drift, it seems to have little effect on another regularity independently noted several years ago by Zulley²⁴⁻²⁸ and Czeisler²³. Figure 2 shows the durations of many sleeps in three independent experiments in conditions of temporal isolation. Sleep duration, plotted vertically, is arranged horizontally according to the timing of sleep onset relative to the temperature rhythm. In Fig. 2a, sleep onset is measured from the previous temperature minimum. In Fig. 2b it is measured from a regression line through a long series of temperature minima found to recur on average every 24.9 h. In Fig. 2c, using the data of Fig. 1, it is measured from a regression line at a 24.33-h period, indirectly inferred to parallel the temperature rhythm. In all cases sleep durations are shorter for sleeps undertaken later—but only up to a point: sleeps undertaken too late after the temperature minimum abruptly become long again.

Our usual 8-h sleeps fall near the middle of this ramp. There is some question about whether the smooth curve one might draw through the descending cloud of dots also rises to connect the cloud's lower end to its upper end: this gap may be a discontinuity typical of rhythmically modulated threshold mechanisms²⁹. There being few sleeps of middling duration (if any) that begin near this critical time, a gap devoid of wake-ups appears in the raster plot (shaded in Fig. 1). There is no corresponding gap devoid of sleep onsets; nor do sleep durations fall into any clear pattern when plotted against sleep onset time; nor can sleep onset time be anticipated with precision comparable to the anticipation of wake-up timing implicit in Fig. 2. Sleep onset and wake onset seem to be governed by different principles.

Mathematical allegories

A variety of mathematical models have been postulated to describe the regularities noted above^{11,13,18,29-34}. For the present they remain more nearly computational metaphors than testable deductions from physiological hypothesis. However, the more sophisticated among them (see, for example, refs 18, 30) may soon prove useful in disentangling the relative contributions of various possible cues to entrainment of human body clocks. Without such cues, and without their somehow perturb-

ing the mechanisms of our internal clocks, we could not keep our habits synchronous with the day/night cycle. Such cues may include mere knowledge of time of day^{8,9,13,35}, light/dark transitions (ref. 36 p. 20, and refs 32, 37–40), compulsory sleep itself^{11,33,36,37,40–44}, food and stimulants^{44–48}, or even a young man's delight to receive illicit daily letters from a young woman lab assistant while the professor was out of town (ref. 11 p. 36, and R. Wever, personal communication).

The supra-chiasmatic nuclei

The physiological sources of circadian timing seem to lurk somewhere in the central nervous system^{49–54}. Control of sleepiness/activity timing in mammals probably resides in a pair of tiny structures called the suprachiasmatic nuclei (SCN) in the hypothalamus, astride the crossing of the optic nerves^{55–59}. Only last year was an anatomical locus presented as the SCN in man⁶⁰. Single cells in this region respond to visible light in rough proportion to the log of light intensity striking the eyes^{61–64}. (The primary visual areas, in contrast, respond mainly to spatial and temporal gradients of light intensity.) This absolute intensity information is conveyed along the retinohypothalamic tracts, identified in a wide variety of mammals (including primates but not yet in man). They start in the left and right retinal ganglion cells and ramify (with mixing) in the left and right SCN⁶⁵. Stimulation of this pathway by light or by electrical impulses does alter the firing of SCN neurones^{61–63,66}. The aggregate firing of SCN cells increases and decreases 10-fold with circadian period in ostensibly constant conditions (ref. 67 and S. T. Inouye and H. Kawamura, personal communication).

In rats the metabolic rate of SCN tissue, assayed by 2-deoxyglucose uptake, also continues rhythmically in constant conditions^{68–70}. An island of SCN isolated from its neural surroundings in the rat still shows a persistent circadian rhythm of neural activity, while similar rhythmicity no longer occurs elsewhere in the brain⁶⁷. It follows that if there is no biochemical or thermal rhythm impinging on the neurally excommunicated SCN, then the SCN in this species is indeed a self-contained oscillator.

Combining observations on rats and monkeys, we might conclude that sleep/wake timing is indeed governed by at least two independently competent clocks. But no one yet knows whether both function in any one species, nor in particular, whether they do in man.

Artificial light: too dim to cue human body clocks?

Our internal clocks normally remain quite predictably synchronized to the 24-h period of environmental cues (and therefore also to each other). The question of what constitutes a compelling cue is obviously of vital importance and is the subject of vigorous experimental investigations. The answers differ significantly from one species to the next. Bright light may be a universal cue; its synchronizing role has been doubted only in the case of man.

Activity in the SCN suppresses release of melatonin from the pineal gland; so does visible light, presumably acting through the SCN. A melatonin secretion rhythm seems to free-run in synchrony with core temperature (A. J. Lewy *et al.*,

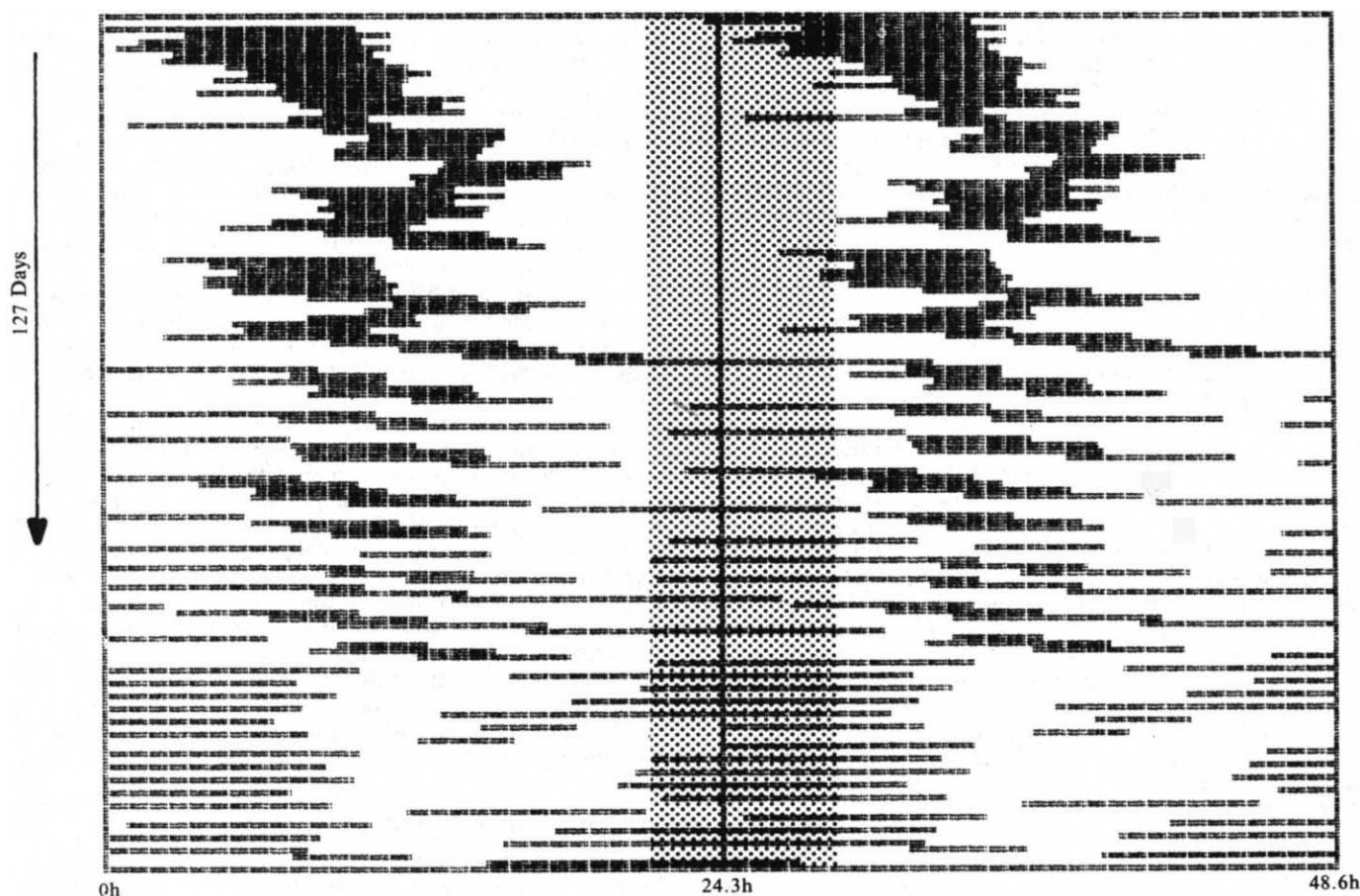


Fig. 1 Data^{21,22} of digitized and reassembled double-plotted raster format following Czeisler²³. Sleep time is indicated by a black line from left to right, with successive 24.3-h intervals stacked from top to bottom. This diagram is really a cylinder of 24.3-h girth along which alternating intervals of sleep and waking are plotted along a helically wound time axis. It is slit open and laid flat for the publisher's convenience; the whole picture is replicated to the right, shifted up a line, to exhibit its continuity across the arbitrary slit line. Notice the shaded range of phase in the 24.3-h cycle when spontaneous wake-up seldom occurs. There is no corresponding range devoid of sleep onsets.

in preparation). The immediate and immediately reversible effect of light on melatonin release thus provides one assay (as yet uncalibrated) for the impact of light on the sleepiness/activity timer.

Quantification of this impact has been sorely needed since the discovery¹³ that in certain conditions light may be a less effective synchronizer than social stimuli (in man)^{11,36,37}. Perlow *et al.*⁷¹ showed that room light inhibits melatonin secretion in primates. Do the several studies showing lack of comparable impact in humans reveal a basic neuro-anatomical or physiological change unique to humans? Richter⁷² presents these speculative matters from a rather different viewpoint, that people override their internal clocks with the aid of artificial light, supposing that artificial light is as good as sunlight for these purposes.

Lewy *et al.*^{73,74} provide a first quantitative estimate of the impact of light in man by monitoring plasma melatonin during exposure to light of various intensities. The results suggest instead that humans are not altogether insensitive but rather only much less sensitive to dim light than are the primates and nocturnal rodents examined to date. In particular, intensities (about 500 lux) comparable to those of indoor artificial illuminants used in experiments throughout the past decade have little or no effect on melatonin secretion. The light/dark alternations used in those experiments may have been dark/dark so far as the sleepiness timer is concerned.

Intensities of about 2,500 lux (more like outdoor sunlight) are effective in man. If similar exposures are needed to phase-shift the circadian rhythm, it could clarify much of the debate over man's entrainment more by social stimuli than by the light/dark cycles that cue every other organism. It also suggests an understanding of Klein and Wegmann's observation⁷⁵ that the symptoms of 6 hours' jet lag persist longer in transatlantic passengers who retire to their hotel rooms to sleep it off, than in others imposed upon to go outdoors⁷³.

Therapy for mood and sleep disorders

In some individuals melatonin secretion is suppressed by dim light. Lewy *et al.*⁷³ report suppression by as little as 500 lux incandescent light in four subjects who are peculiarly susceptible to manic-depressive alternations. This observation enhances interest in recent conjecture^{74,76-80} that fluctuations of mood, including manic-depressive alternations, may be caused by abnormal mutual phasing of circadian rhythms. Wehr *et al.*⁷⁸ have found that by deliberately advancing the sleep/wake rhythm by 6 h relative to the temperature rhythm, they could sometimes abort depressive episodes in one patient. More recently in another patient, Lewy *et al.*⁸¹ precociously terminated recurrent winter-time depression by artificially lengthening the photoperiod.

Phase relations between sleep and core temperature can also be quite different in individuals who for some reason are not normally responsive to the usual synchronizing cues⁸². Among sightless individuals, those who do entrain show diverse phase angles relative to the civil and solar day^{83,84}. Some sightless individuals, some recluses and older people living indoors with little social contact, and perhaps individuals with stronger circadian clocks, sometimes retire and rise later and later every day like the tides. Eventually, pursuing their solitary interests by night and sleeping by day, 180 degrees out of phase with surrounding society, they drift still later into synchrony again after another 2 weeks or so⁸⁵⁻⁸⁸ (see Fig. 3 for an example).

All subjects fail to synchronize to scheduling at too short or too long a period relative to their own internal clocks. Wever⁸⁹ has exposed subjects in his temporal isolation facility to gradually lengthening (or shortening) days. As their activity cycles shift with respect to the light/dark cycle and their temperature cycles shift with respect to their activity cycles, they gradually become 'early birds' or 'night owls'. This becomes noticeable subjectively only when their usual timing of waking or retiring transgresses a boundary of daylight. However, when

the driving period becomes too extreme (beyond 22 or 27 h), both internal and external synchronization fail: neither core temperature fluctuations nor sleep/wake alternations follow the light/dark cycle, nor do they remain entrained to each other. Curiously, the subject remains unaware of any change

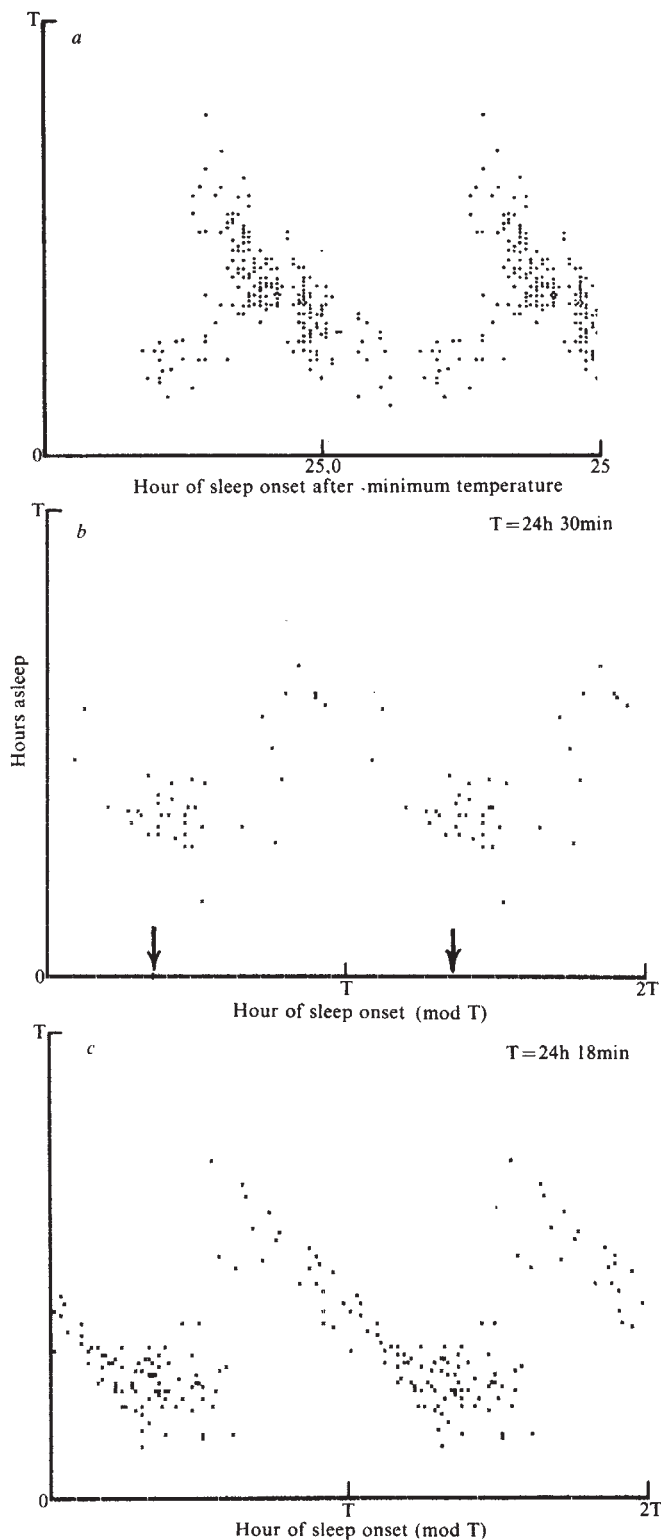


Fig. 2 a, Human sleep durations plotted against circadian phase (from ref. 27) as described in the text. b, As in a, with PRO1 data from ref. 23, as described in text; the arrows mark approximate temperature minima. c, As in a and b but data from refs 21, 22, subject J.C. digitized and reprocessed following Czeisler²³. The blank line inserted after 7 weeks represents transition to continuous dim light for the final 80 days. Note that the vertical and horizontal scales are not identical here.

within himself: he just thinks that the room lights are being operated capriciously^{11,36,89}.

Some kinds of insomnia, previously dismissed as personal idiosyncrasy, seem to reflect treatable aberrations of the circadian clock and/or the sleep/wake system. A simple manoeuvre is reported⁹⁰⁻⁹² to cure the 'delayed sleep phase syndrome', estimated to afflict hundreds of thousands of people. Some people's sleep/wake rhythm, subject to rhythmic driving both by the internal temperature oscillator and by an external civil day, adopts an inconvenient phase relative to the civil day. In the cases that come to clinical attention, both sleep and waking are tenaciously delayed about 6 h relative to what is usual for most people. This makes it virtually impossible for them to hold any job that requires alertness in the morning. However, by delaying three more hours each day for 6 days, one eventually arrives at the desired schedule and it remains stable long after termination of the phase-shifting therapy (Fig. 4). However, the problem recurs after working late, travelling too far westward too abruptly, or otherwise losing yet 6 hours more. Then another 18-h delay must be arranged^{90,91}. It would appear that such individuals can travel abroad only by remaining on a home-base schedule during a brief excursion, or, if they must synchronize to the civil day abroad, by circumnavigating and crossing the International Dateline westward.

Daylight savings, jet-lag and shift work

A small phase shift of this sort has been imposed on hundreds of millions of people twice annually since the implementation of Benjamin Franklin's plan for daylight-saving time. The possible consequences were never considered and are not fully known. Monk and Aplin⁹³ recently found that the time of retiring adjusts immediately but that the time of awakening (or



Fig. 3 In the same format as Fig. 1 but with 24.0-h girth, a recording of more orderly alternations of sleep and waking in a non-blind individual who, nevertheless, does not synchronize to the 24-h world. His own period waxes and wanes (the slope of the sleep-onset border varies) as his phase relation to the 24-h world changes (from ref. 88). Note again the shaded zone of no-waking in this man's internal period, roughly corresponding to his temperature minimum.

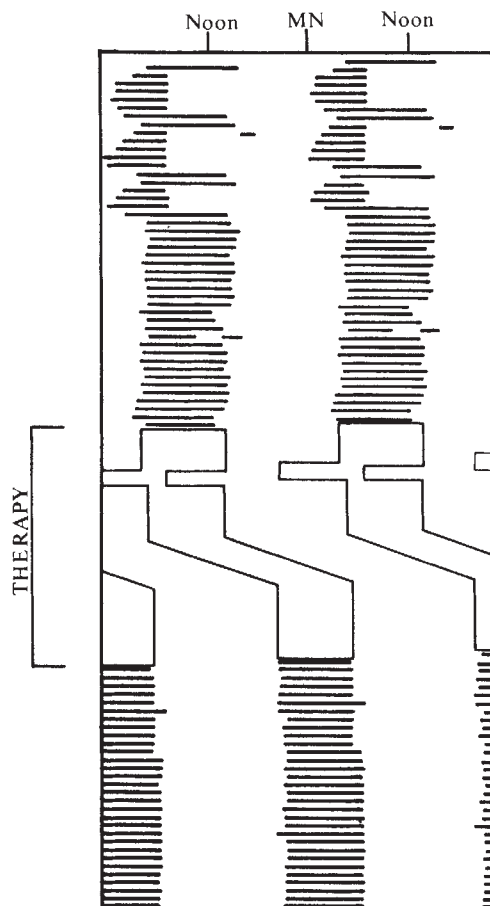


Fig. 4 The format is as in Fig. 3. Sleep is indicated by a black bar in each of 110 stacked days. During therapy, sleep was confined within the zigzag channel. It retains the new timing with respect to the civil day. (Adapted from ref. 91.)

duration of sleep, see Fig. 2) needed a week to adjust fully. The advancing (springtime) shift was somewhat more difficult than the delaying (autumnal) shift, as might be expected *a priori* for a species whose internal clock already has a somewhat longer period than the natural day. Much the same happens when we travel either east or west. Travel by air compresses the phase shift into a single day. After transmeridian flights, about one person in four reports some indisposition associated with resynchronization⁹⁴. The abundant literature of jet-lag seems internally contradictory in many respects, and therefore suggests that individuals vary enormously in their reactions to diverse rescheduling stimuli. Recent reviews⁹⁴⁻⁹⁸ agree that internal phase relations remain abnormal for up to a week. Nearly all flight experiments indicate substantially quicker adjustment after westward (delaying) flights across as many as nine time zones, than after comparable eastward (advancing) flights. The latter also result in a depression of temperature-rhythm amplitude for several days. Duration of flight, whether outbound or homebound, the time of departure and change in wake duration do not matter for the subsequent course of resynchronization.

Both in laboratory isolation units and outside, the diverse physiological and psychological rhythms readjust at their diverse individual rates—even with some advancing and some delaying, most commonly when crossing 9 to 12 time zones^{41,94}. In free-running conditions of temporal isolation, subjects do not perceive the jet-lag-like dissociation of their own internal rhythms. Wever^{11,99} reports that subjects even feel better during the prolonged dissociation he calls internal desynchronization. These observations conflict with universal expectation that

derangement of internal timing should cause discomfort or damage. I know of no unequivocal demonstration that this internal dissociation in itself causes trouble apart from the inconvenience of trying to sleep, eat or think quickly at times when either local society or one's own body is not ready for it. This latter discomfort is experienced by individuals who are out of synchrony with society but still internally synchronized. This can be important: shift workers include air-traffic controllers, nuclear submarine and power-plant operators, emergency-room doctors and intensive-care unit nurses. Greater error rates have been measured during shift work^{94,100,101}. Individuals whose temperature exhibits larger daily excursions tend to take longer to adjust to each shift^{41,99,102}. This suggests itself as a selection criterion for individuals required to cope with frequent changes of scheduling.

What is the best procedure for hastening adaptation to a new schedule (assuming this is a good thing to do)? Advice seems unreliable but the best guess may be just to maximize the

contrast between our circumstances day and night with respect to light/dark, meals and social factors that provide synchronizing cues. My personal impression, based on no more than conversation with other travellers during about fifty Atlantic crossings, is that for some people direct sunlight helps a lot.

The timing of sleep and wakefulness is only one facet of the intricate patterning of temporal contrasts that pervade human physiology. The medical profession is beginning to find ways to incorporate recent understanding of the predictable ups and downs of metabolism, the large excursions of hormone titre, drug susceptibility and plasma ion balance that previously limited the effectiveness and safety of routine procedures^{103,104}.

I am indebted for financial support during this survey to the NSF, the Institute for Natural Philosophy and the USAF School of Aerospace Medicine; and for hospitality to George Oster and the University of California at Berkeley during sabbatical leave from Purdue University. I thank the many workers here cited who freely shared with me their time and unpublished data.

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ARTICLES

An explanation of $^{13}\text{C}/^{12}\text{C}$ variations in tree rings

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Variations in the $^{13}\text{C}/^{12}\text{C}$ ratio in trees are examined in the light of a simple expression relating the relative isotope composition of plant material, δ_p^{13} to δ_a^{13} , the atmospheric isotope value, c_a , the atmospheric CO_2 concentration and c_i , the internal concentration of CO_2 in leaves. The expression gives good agreement with δ_p^{13} measurements where independent information on c_i exists, such as seasonal growth, growth low in the canopy and in conditions of low humidity. The expression provides possible explanations for two previously unexplained phenomena: the absence of anticipated changes due to fossil fuel-induced changes in δ_a^{13} , and regional differences in δ_p^{13} trends.

THE isotopic composition of carbon stored in the growth rings of trees may represent a record of $^{13}\text{C}/^{12}\text{C}$ variations in atmospheric CO_2 , or of physiological responses to environmental changes, or a combination of both. In the first category, several authors¹⁻⁴ have attributed a decrease in $^{13}\text{C}/^{12}\text{C}$ in tree rings over the period 1850–1950 to an increasing component of ^{13}C -depleted CO_2 , released into the atmosphere by increasing fossil fuel combustion, and/or by deforestation and expanding agriculture. In the second category, others⁵⁻⁹ have sought an explanation of $^{13}\text{C}/^{12}\text{C}$ variations in tree rings through correlations with temperature. Here there is normally an assumption of constant $^{13}\text{C}/^{12}\text{C}$ in the atmosphere, and a common, temperature-sensitive metabolic process within plants is implied. In the third category, Mazany *et al.*¹⁰ assumed $^{13}\text{C}/^{12}\text{C}$ in air to be influenced by respiration from surrounding vegetation, which in turn responds to environmental factors such as temperature and water availability. As in the first category Mazany *et al.* assumed constant fractionation in the carbon flow from the atmosphere to the biosphere.

We investigate here the applicability to tree-ring studies of a new, quantitative expression¹¹, relating the carbon isotope ratio in those plants having the most common C_3 mechanism of photosynthetic carbon fixation to the physiological properties of a leaf. When applied to tree-ring sequences corresponding to the period of industrialization, the fractionation expression carries implications concerning the response of trees to increasing levels of CO_2 in the atmosphere.

Carbon discrimination in plants

An expression¹¹ for δ_p^{13} of the photosynthate in a C_3 plant is approximated in the form

$$\delta_p^{13} = \delta_a^{13} - a - (b - a)c_i/c_a \quad (1)$$

where δ_a^{13} is the fractional difference between $^{13}\text{C}/^{12}\text{C}$ in air surrounding the leaf and that in a reference material; c_a and c_i are the CO_2 concentrations in the external atmosphere and leaf intercellular spaces, respectively. Furthermore,

$$c_i = c_a - A/g \quad (2)$$

where A is the rate of CO_2 assimilation by the plant and g is the conductance of the boundary layer and stomatal pores to the diffusion of CO_2 . The diffusivity of $^{13}\text{CO}_2$ in air is 4.4% less than that of $^{12}\text{CO}_2$, and this is the value of the constant a . The value of b is determined by the isotopic discrimination of

the ribulose-1, 5-bisphosphate carboxylase enzyme, thought to be the main source of fractionation in C_3 plants¹², with magnitude $\sim 30\%$ (ref. 13).

In the derivation of equation (1) the CO_2 concentration drop from the intercellular spaces to the chloroplasts is assumed to be small, and intrinsic fractionation in photo- and dark-respiration steps is assumed negligible. In its application to tree rings, the fractionation associated with translocation, and with the conversion of the photosynthate to other chemical fractions such as cellulose or lignin, is assumed to be relatively small and not influenced by environmental factors. In the application of equation (1) in this article we assume that b is constant.

Qualitatively, this model is quite similar to those of Park and Epstein¹², Troughton¹⁴ and Grinstead¹⁵. Progress towards a quantitative model has also been made independently^{13,16} but the ease of application of equation (1), is a significant advantage in interpreting carbon isotope behaviour in plants. It is supported by the range of δ_p^{13} values observed in C_3 plants and by a correlation between δ_p^{13} and c_i/c_a in these species¹⁷. Further support is drawn from measurements of δ_p^{13} and c_i/c_a following water stress¹⁸ and of the temperature^{19,20} and humidity²¹ dependences of δ_p^{13} in growth chamber experiments.

Paraphrasing¹⁹, factors which reduce CO_2 assimilation rate through effects primarily on the mesophyll capacity for photosynthesis (for example, very low light intensities, and deficiencies of certain mineral nutrients) will increase c_i/c_a and reduce δ_p^{13} ; factors which reduce CO_2 assimilation rates primarily through reduction of supply of CO_2 through the stomata (for example, large vapour pressure deficits) will decrease c_i and increase δ_p^{13} . Note that in some situations a change in an external parameter may affect both A and g in equation (2) with no marked effect on c_i/c_a and thus δ_p^{13} (ref. 19).

Discussion

The possible relevance of equation (1) is explored with reference to observations of a number of reported phenomena. Note that because relative rather than absolute δ^{13} values are discussed, the term δ_p^{13} is used at different times in reference to different material (such as cellulose, whole wood, and leaves). We discuss (1) the seasonal variation in δ_p^{13} ; (2) the 'juvenile' effect in δ_p^{13} ; (3) correlations between δ_p^{13} and regional ring width, temperature and precipitation; (4) variations of δ_p^{13} within a tree; (5) the absence of a fossil fuel-induced depletion of ^{13}C in Tasmanian trees; (6) regional differences in long-term δ_p^{13} trends.

Seasonal variations in δ_p^{13}

Lowden and Dyck measured a 2–5% decrease in δ_p^{13} values in both maple leaves and grass collected from an exposed site in Quebec during May to September. The seasonal (and diurnal) variation in δ_a^{13} in the free atmosphere obeys a simple atmosphere biosphere mixing relationship^{23–25}. With typical ≥ 10 p.p.m.v. concentration decreases from May to September at high northern latitudes, this implies a δ_a^{13} increase of $\sim 0.5\%$, far smaller and in an opposite sense to the seasonal change in δ_p^{13} . Lowden and Dyck hypothesize that the δ_p^{13} changes might be a direct consequence of changing photosynthesis to photorespiration ratios.

Equation (1) is a quantitative description of such processes. Whilst no information has been located on the seasonal variation of c_i in trees, there is ample evidence in measurements on crop plants of c_i increasing with age from peak photosynthetic rate to near senescence^{26–30}. Typical increases are 20–50 p.p.m.v., which in equation (1) for typical values of c_a , δ_a and b , imply δ_p^{13} decreases of 2–4‰, in close agreement with the values of Lowden and Dyck in both leaves and grass.

During initial leaf development A will be low and c_i high; that is over the full growth season we might expect a rapid decrease in c_i , a levelling off, followed by the slow increase to senescence³⁰. If this occurs in *Pinus radiata*, then measurements of 1–2‰ changes in δ_p^{13} within a ring of a New Zealand specimen³¹ can be readily explained by equation (1). These indicate increasing δ_p^{13} early in the growth season (1/6th of a ring width), with high or slowly decreasing δ_p^{13} over the middle (4/6ths of a ring), and a very low δ_p^{13} in the late wood (1/6th of a ring). The effect is measured in both cellulose and lignin, confirming a mechanism early in the sequence of CO_2 assimilation. The trend of the δ_p^{13} change with respect to season is confirmed by measurements on early and late wood from Australian *Athrotaxis selaginoides*³². (At these latitudes c_a and δ_a^{13} in the free atmosphere are practically constant throughout the growth season^{33,34}). In both cases the magnitude of the δ_p^{13} change of 1–2‰ is perhaps too large to be explained by temperature dependent fractionation process, or by local modification of δ_a^{13} , but quite compatible with observed seasonal changes in c_i .

The juvenile effect in δ_p^{13}

Steadily increasing δ_p^{13} values with time, often observed in the innermost rings of a tree^{4,15,35,36}, have been attributed^{4,37} to reassimilation of respired CO_2 which is retained within the plant canopy. But this explanation has been questioned for trees which grew in relative isolation, and an age-related physiological factor suggested^{15,35}.

A step increase in δ_p^{13} accompanying a step increase in ring width³⁸ during early growth years, together with no δ_p^{13} increase in a well-exposed island tree³⁸, argue against an intrinsic age-related effect, and are qualitatively compatible with a 'canopy-effect'.

The close proportionality between δ_a^{13} and c_a values observed in forest environments by Keeling^{24,25} can be used to predict c_a levels some 40 p.p.m.v. above ambient, which must be maintained on average during photosynthesizing periods, to produce the typical 2‰ magnitude of the 'juvenile effect'. Studies in a coniferous forest³⁹ (and O.T. Denmead, unpublished) and in a tropical rain forest⁴⁰ show that during daytime CO_2 in air is generally depleted by < 4 p.p.m.v. through most of the stand; higher concentrations only occur close to the forest floor. Even in dense forests δ_a^{13} is similar to that in background air^{24,25}. Periods of maximum photosynthesis generally coincide with periods of maximum instability and convective mixing in the atmospheric surface layer, whereas the value of 40 p.p.m.v. above ambient represents night-time conditions of extreme stability and dark-respiration in the Keeling data, and far exceeds any daytime value. Normal selection of trees for δ_p^{13} analysis minimizes those situations of extreme atmospheric stagnation which might favour significant ^{12}C cyclic enrichment in trees³⁷.

While soil respiration may contribute to low δ_p^{13} low in the canopy through δ_a^{13} in equation (1), changes in c_i/c_a offer an alternative, or at least a contributory, explanation. At low irradiances rate of assimilation, A , and to a lesser extent, stomatal conductance, g , are reduced. The net result is that c_i increases⁴¹. In the extreme, as the irradiance drops near that level at which the photosynthetic rate is zero, c_i approaches c_a , the concentration of CO_2 outside the leaf. On these grounds we expect low δ_p^{13} values at low irradiances.

Measurements on wheat plants grown in well-ventilated controlled chambers under shade cloth (in $220 \mu\text{E m}^{-2}\text{s}^{-1}$) when compared with similar plants grown in light levels of $640 \mu\text{E m}^{-2}\text{s}^{-1}$ show a 2‰ reduction of δ_p^{13} (ref. 20), strongly suggesting that low light levels do produce δ_p^{13} changes of the sign and magnitude of the observed juvenile effect.

Shading is unlikely to produce an explanation for juvenile ^{13}C depletion in isolated trees. However, there are other external factors which affect the mesophyll capacity for photosynthesis and which could well vary during early tree development, for example, certain nutrient deficiencies, so that equation (1) provides a promising starting point for further research.

δ_p^{13} and ring width, temperature and precipitation

The seasonal variation interpretation, plus interpretation of δ_p^{13} versus temperature results from growth chamber measurements^{19,20}, associate more positive δ_p^{13} with faster assimilation rate, A . Correlations of δ_p^{13} with ring width by Mazany *et al.*¹⁰ and Tans and Mook⁴² associate more positive δ_p^{13} with narrow rings and thus presumably slower assimilation rates.

The general response of trees in arid areas is for ring widths to be wide in wet, cool years and to be narrow in warm, dry years; extreme water stress can cause stomatal closure and reduced CO_2 uptake as shown by Fritts⁴³. Low humidity has been shown⁴⁴ to reduce g and c_i , so that in conditions of high temperature and low precipitation we could expect narrower rings, lower c_i and, by application of equation (1), higher δ_p^{13} , as seen by Winter and colleagues^{18,21}. Measurements on juniper leaves and twigs from a wide area of arid Arizona, in which more negative δ_p^{13} are associated with lower growth temperatures and higher precipitation⁴⁵, are compatible with this.

A similar argument can be applied to the Mazany *et al.*¹⁰ trees from the same area; however, they demonstrate a striking improvement in the δ_p^{13} versus ring-width index correlation when a regional rather than individual ring width index is used. This seems to be their major reason for favouring an explanation in terms of regional modification of the atmosphere by respired CO_2 (that is, good conditions are reflected in vigorous regional growth, more respiration and an atmosphere with depleted δ_a^{13}). The rapidity with which CO_2 mixes in the atmosphere, particularly during photosynthesizing hours and in sparsely vegetated areas^{24,25,46,47} would seem to invalidate an explanation involving significant regional modification of c_a , δ_a^{13} values. In the context of equation (1), the higher correlation with regional indices requires that changes in average c_i are primarily determined by contemporary regional conditions whereas many factors are known to determine individual ring widths. The fact that δ_p^{13} in the Dutch trees correlates inversely with ring width and directly with temperature and inversely with precipitation⁴², as inferred for the Arizona trees, is suggestive that similar influences are at work.

A recent summary of δ_p^{13} versus temperature coefficients indicates considerable complexity⁴⁸. In general, the δ_p^{13} response in tree rings to environmental parameters such as temperature and precipitation might be expected to be complex if δ_p^{13} is determined from equation (1). For example, growth chamber measurements on tree seedlings show how both positive and negative δ_p^{13} versus temperature coefficients might be obtained, depending on whether the change is below or above the temperature corresponding to peak photosynthetic

activity^{19,20}, and the optimum temperature is different for different species. Thus there may be both a species and a range dependence. Added to this will be quite non-linear influences resulting from stress, the most obvious being stomatal response to extreme hot dry conditions. This places great emphasis on site and species selection if δ_p^{13} in trees is to be used for environmental reconstructions.

Variations in δ_p^{13} within a tree

Several authors have drawn attention to variations of δ_p^{13} around the circumference of a ring^{4,10,38,42} of magnitude 1–4‰. Again it is difficult to explain the magnitude of this effect in terms of local modification of δ_a^{13} —previous measurements of δ_a^{13} versus c_a (refs 24, 25) require large CO₂ gradients of 20–80 p.p.m.v. to be maintained across a tree for the major portion of the growth periods. It is especially difficult as these trees have generally been selected from positions of relative isolation.

For the same reason large differences due to a cyclic enrichment process³⁷ are difficult to envisage. The main evidence presented by Keeling for the cyclic enrichment process, effectively a comparison of maximum daytime δ_a^{13} values with maximum night-time c_a values at various forest sites, can be explained by the seasonal variation in the large-scale atmospheric δ_a^{13} values.

Variations in stomatal conductance, g , for example due to differences in structure and shading in leaves⁴⁹, or variations in leaf assimilation rate, A , due to shading²⁰, appear to offer a more realistic explanation through equations (1) and (2). For example, even though Wong *et al.*⁵⁰ show that c_i is approximately constant in *Eucalyptus pauciflora* over a range of irradiances above 250 $\mu\text{E m}^{-2}\text{s}^{-1}$, the small changes observed are sufficient to imply δ_p^{13} changes of ~1‰.

No fossil-fuel depletion of ^{13}C in Tasmanian trees

Cellulose from eight Tasmanian trees³⁸ exhibits a practically constant δ_p^{13} with time over the period 1750–1970. This is in contrast to the predicted decrease of 0.7–0.8‰ (refs 42, 51). There is even greater contrast if an additional large amount of CO₂ has been released as a result of forest clearing and agricultural practices³¹. It does not reflect direct observation of a δ_a^{13} change in the atmosphere⁵². It also contrasts with a summary of most reported Northern Hemisphere δ_p^{13} measurements⁵³.

The eight Tasmanian trees represent two species, and a wide range of environments (small island to extremely exposed mountain ridge) but with all sites sharing desirable features in the context of monitoring global atmospheric CO₂. The areas are remote from large-scale industry and experience persistent winds off the southern ocean, enhancing mixing. The seasonal variation in CO₂ is small and temperatures are stabilized by the surrounding (particularly upwind) oceans. The very high and persistent rainfall (typically in excess of 2,000 mm yr⁻¹) implies minimum water stress with its corresponding effects on fractionation. In a forest situation the selected species typically have most of their foliage at or above canopy level.

The absence of a δ_p^{13} trend which reflects the anticipated δ_a^{13} trend in any one of the eight trees, requires an effect operating on a regional scale (≥ 100 km) of similar magnitude but opposite sign to the fossil fuel signature in the atmosphere. Equation (1) provides a possible solution without recourse to complete coincidence. While the δ_a^{13} decrease due to fossil fuel release is associated with an increase in c_a , the effect of increasing c_a in the equation, all else being constant, is to increase δ_p^{13} .

Physiological data on c_i/c_a in trees is scarce, and over the 50–100 year time scale, is non-existent. The strength of the present argument rests with the feasibility of the mechanism, when no alternative explanations exist. Even those physiological experiments which suggest constancy of c_i/c_a in some plants over short time scales¹⁹, are of an accuracy which do not exclude

the small changes necessary to reconcile the Tasmanian δ_p^{13} measurements with the anticipated δ_a^{13} changes due to fossil fuel combustion.

For example, combination of the predicted δ_a^{13} curve^{42,51} with the Tasmanian mean curve plus equation (1) implies

$$[c_i/c_a]_{1970} \approx 0.9[c_i/c_a]_{1870}$$

and thus

$$[c_i]_{1970} \approx [c_i]_{1870}$$

In other words the trees have, to a first approximation, maintained a constant c_i . This conclusion does not depend on the value of b , and depends only weakly on the δ_p^{13} value used. The simplistic application of equations (1) and (2) implies either reduced stomatal conductance, g , or increased assimilation rate A over the 100 years of increasing CO₂ levels. In view of the abundant water availability, the latter is favoured.

There are many complexities in the real situation which must be better understood before the model for fractionation and the tree records can be used with confidence in this fashion. However, we have illustrated a possible sensitive means of probing physiological behaviour over long time scales.

Regional differences in long-term δ_p^{13} trends

A composite δ_p^{13} curve from 26 European and North American trees shows a decrease of almost 2‰ from 1850 to 1975, which includes a slight increase between 1940 and 1960 (refs 4, 53). Several Northern Hemisphere individual trees^{2,3} have been reported with similar δ_p^{13} decreases up to around 1940–50, all in marked contrast to the practically constant δ_p^{13} in the Tasmanian trees from 1850 to 1970.

Considerable caution is required in attributing these differences in δ_p^{13} to environmental differences on a hemispheric scale. For example, the mean of four Dutch trees⁴² decreases between 1860 and 1940 by no more than 0.5‰, with an increase of almost 0.4‰ from 1940 to 1970; overall these are not significantly different (in view of the scatter between individual trees) from the Tasmanian trees. But the one other reported Southern Hemisphere tree-ring result¹ is very different from the Tasmanian results with a steady 1–1.5‰ decrease from 1890 to 1960.

The predicted change in global δ_a^{13} due to fossil fuel combustion of around 0.7–0.8‰ (refs 42, 51) from 1750 to 1970 is equally incompatible with the 2‰ change of Freyer⁴ as it is with the flat Tasmanian record. In both cases the differences from the predicted curve are too large to be explained by measurement error. A large additional release of CO₂ around 1860 associated with an 'agricultural explosion' has been postulated to explain the larger δ_p^{13} trends^{4,31}.

Use of an 0.032‰ p.p.m.v.⁻¹ biosphere-atmosphere mixing relationship observed⁵⁴ between 1956 and 1978 as an upper limit to that applicable over the past 200 yr can be used to predict 1750 levels of atmospheric CO₂ considerably lower than the 270–290 p.p.m.v. typically employed in global carbon budgeting models (G.I. Pearman and I. Enting, personal communication). More realistic mixing over the 200-yr time scale comes from such models^{42,52}, which include realistic air-sea fractionation factors, and these suggest a relative insensitivity of δ_a^{13} to biospheric release. Thus it is not obvious that the postulated explanation for the large δ_p^{13} trends is valid.

The maximum latitudinal difference in δ_a^{13} that can be maintained over decades is expected to be small. For example, the current difference in annual average CO₂ concentration of 3–4 p.p.m.v. from pole to pole³¹, if assumed due to fossil fuel combustion, represents a 0.1–0.2‰ difference in δ_a^{13} . Keeling *et al.*⁵⁴ measured a difference between North America and South Pole of <0.1‰ in 1978, although results of Kroopnick *et al.*⁵⁵ in 1970, when corrected for seasonal effects indicate a 0.5‰ difference over a similar latitude range. A recent two-dimensional and seasonal model of global carbon exchange

(refs 33, 56–58 and P. Hyson, personal communication), estimate a total change, 1850–1970, of $\sim 0.2\%$ between 50°N and 50°S . Thus even if the Freyer⁴ results do reflect a global δ_a^{13} change, the conflict with the Tasmanian δ_p^{13} trends is about twice that discussed above. It is true that both cannot represent large scale δ_a^{13} changes.

It is equally unrealistic to suggest that regional or sub-canopy modification of δ_a^{13} could explain the differences. This is because of the small magnitude of daytime δ_a^{13} differences from background air in even quite dense forests^{24,25}, and the wide variation in ages, exposures, and species, of trees showing common trends³⁸. The only possibility of reconciling all observations, seem to be in systematic variations in the fractionation of atmospheric CO_2 as it is assimilated by trees.

Drawing on examples and discussion above, possible examples of environmental influences on the fractionation pro-

cess in trees which operate locally, but on decadal to century time scales, include: (1) shading in developing forests, where individuals have significant subcanopy foliage; (2) nutrient depletion; (3) changes in assimilation rate resulting from regional temperature trends, or from interactions with changing atmospheric pollutant levels; (4) changes in stomatal behaviour resulting from trends in atmospheric pollutants or humidity.

For tree-ring sequences over the past 50–100 yr it can be argued that δ_a^{13} is known to greater accuracy than c_i/c_a ; using the fractionation expression, measurements of δ_p^{13} may become useful in plant ecophysiology.

We thank Dr G. I. Pearman, Mr P. Hyson and Dr Ian Enting (CSIRO, Division of Atmospheric Physics) for computational work with respect to the carbon budgeting models. Professor C. B. Osmond and Dr Ian Cowan and Dr Roger Gifford offered valuable comments.

Received 7 January; accepted 1 March 1982.

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Function of a tRNA gene promoter depends on nucleosome position

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We have used an in vitro procedure to direct nucleosomes to different sites on a chicken embryo tRNA gene. The results show that transcription of the tRNA gene depends on nucleosome position and in a functional promoter structure, the constant gene sequence TTCGA coincides with the nucleosome middle axis.

EVIDENCE is accumulating that the chromatin structure of expressed genes differs in several respects from non-expressed genes^{1–14}. As genes are associated with nucleosomes regardless of their functional state¹⁴, the recognition of nucleosomes that have become specifically altered requires a higher level of programming such as exposure of a recognition DNA base sequence. The particular location of such a sequence within the nucleosome could serve as the structure that is recognized. Such a nonrandom association of nucleosomes with respect to DNA

base sequence is generally called 'phasing' although this term covers several different phenomena (for review see ref. 15). The limits of long-range phasing of regularly spaced nucleosomes and of sequence-specific nucleosome positioning have been discussed critically by Kornberg¹⁶. It is possible that base-specific DNA binding proteins serve as a phase trigger for regularly spaced nucleosomes. In the assays normally performed, such nucleosomes would display a nonrandom association with the DNA base sequence, unless due to inherent

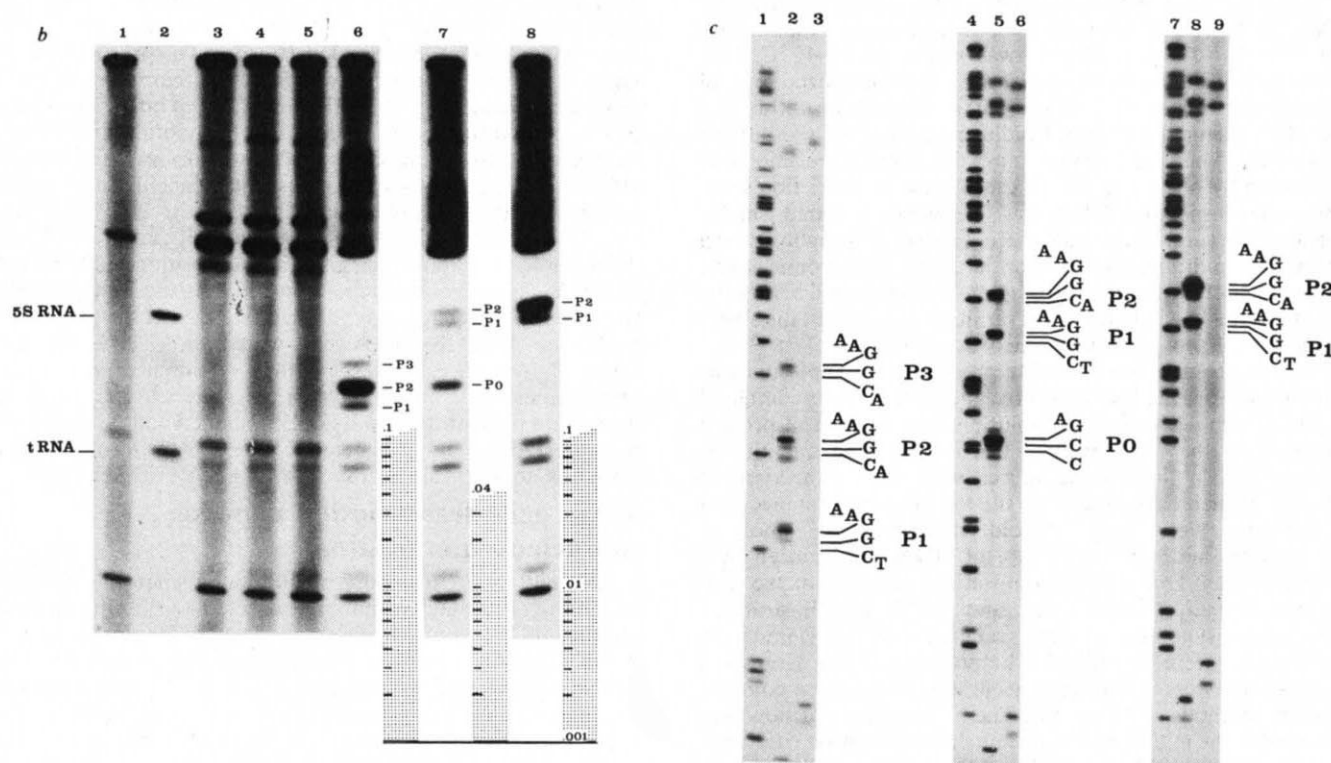
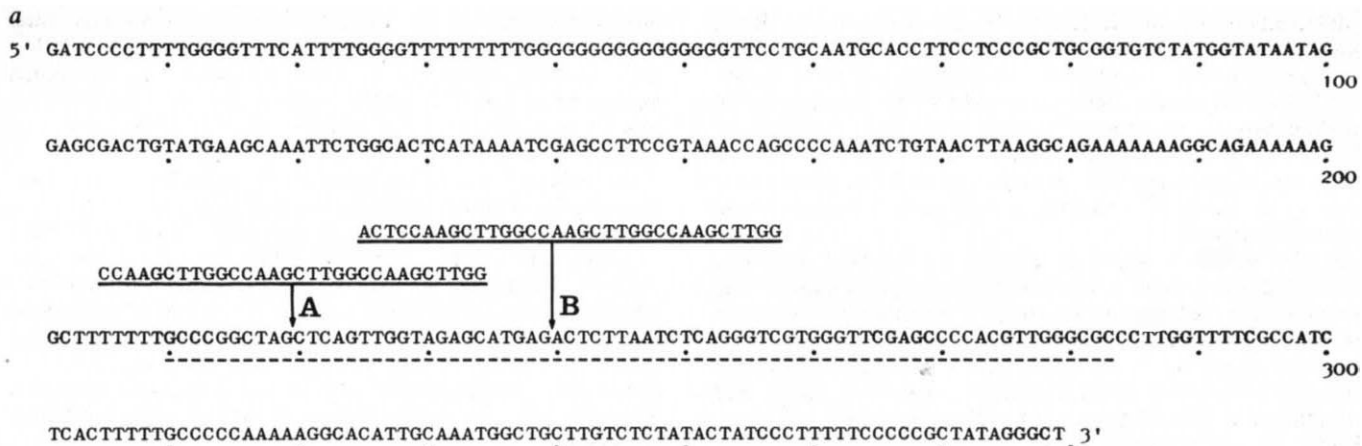


Fig. 2 *a*, DNA base sequence of the 379-bp subclone of a chicken embryo tRNA^{Lys} gene. Broken line represents tRNA structural gene sequence; solid lines, 30-bp palindromic DNA. Vertical arrows labelled A and B indicate type A and type B insertions, respectively. *b*, Transcription of the tRNA^{Lys} gene within randomly positioned nucleosomes. 1, Assembly extract minus sssDNA; 2, chicken embryo tRNA^{Lys} and *E. coli* 5S RNA; 3, isolated DNA lacking 30-bp palindromic insertion (after incubation with assembly extract); 4, isolated DNA with type A insertion; 5, isolated DNA with type B insertion; 6, randomly positioned nucleosomes on DNA without insertion; 7, randomly positioned nucleosomes on DNA with type A insertion; 8, randomly positioned nucleosomes on DNA with type B insertion. The bands representing radioactively labelled tRNA precursors were cut out and quantified by liquid scintillation counting. Efficiencies of transcription (mol tRNA precursor per mol tRNA gene) are indicated by columns on a logarithmic scale shown beside the respective electrophoresis slot. A 25- μ l nucleosome assembly extract was diluted to 500 μ l with 100 mM KCl, 20 mM NgCl, 10 mM 2-mercaptoethanol, 10% glycerol, 50 mM Tris-HCl pH 7.9, and centrifuged at 4 $^{\circ}$ C in a type 25 rotor (Beckman Instruments) at 90,000g for 3 h. The supernatant was pipetted off and immediately 100 μ l of 100 mM KCl, 20 mM NaCl, 2 mM MgCl₂, 10 mM 2-mercaptoethanol, 10% glycerol, 0.5 mM ATP, 0.5 mM CTP, 0.5 mM UTP, 50 mM Tris-HCl pH 7.9, were added and vortexed briefly. Of 0.25 μ g sssDNA used in the assembly extract, at least 70% were recovered (as monitored by the ³²P-label in the 30-bp palindromic DNA) together with ~50 μ g (5%) and ~2 μ g (3%) of residual protein and nucleic acids, respectively, from the assembly extract. 12.5 μ l of 250 μ M [α -³²P]GTP (10 Ci mmol⁻¹) and 12.5 μ l of partially purified RNA polymerase III (0.1 U, 20 μ g protein) were added and incubated at 37 $^{\circ}$ C for 10 min. 'Carrier' plasmid DNA (5 μ g) was added, and nucleic acids were deproteinized and precipitated, then dissolved in 25 μ l of 1 mM EDTA, 10 mM Tris pH 7.4, and restricted with *Hind*III to cut ³²P-labelled DNA (originating from sssDNA) into 10-bp fragments. ³²P-RNA was analysed on 6% polyacrylamide, 7 M urea gels (2 $\times$ 250 $\times$ 450 mm). Chicken embryo DNA-dependent RNA polymerase III was purified as described elsewhere³⁰, and the material obtained after DEAE-Sephadex chromatography was used. *c*, S₁ nuclease hybridization mapping of transcription products. 1, Guanosine-specific partial chemical cleavage (G-ladder) of sense transcription probe from DNA without insertion; 2, randomly positioned nucleosomes on DNA without insertion, sense transcription mapping; 3, as in 2, but anti-sense transcription mapping; 4, G-ladder of sense transcription probe from DNA with type A insertion; 5, experiment IV, sense transcription mapping; 6, expt IV, anti-sense transcription mapping; 7, G-ladder of sense transcription probe from DNA with type B insertion; 8, expt VIII, sense transcription mapping; 9, expt VIII, anti-sense transcription mapping. 1 μ g of sssDNA was used in the assembly extract and transcription was performed in the presence of 0.5 mM GTP instead of ³²P-labelled GTP. After *Hind*III restriction, nucleic acids were precipitated and dissolved in 25 μ l of 80% formamide, 0.4 M NaCl, 1 mM EDTA, 50 mM PIPES, pH 6.8 containing 1 μ g of ³²P-labelled DNA hybridization probe. Hybridization was at 55 $^{\circ}$ C for 16 h. 250 μ l of ice-cold 200 mM NaCl, 1 mM ZnSO₄, 25 mM Na-acetate pH 4.5 and 10 U of S₁ nuclease were added and incubated at 37 $^{\circ}$ C for 3 h. Nucleic acids were precipitated (5 μ g of carrier tRNA were added) and analysed on a 10% sequencing gel. Probes for S₁ nuclease hybridization mapping were prepared from plasmids pBR322 N4 *Bam* *Sau*A and pBR322 N4 *Bam* *Sau*B, containing the 379-bp fragment of nucleosomal DNA (see Fig. 2a) with a type A and type B insertion, respectively.

solubilization of class I, II and III DNA-dependent RNA polymerases from chicken embryos³⁰. A nucleosome monomer fraction comprising a mixture of core particles and nucleosomes (the histone H1 content being reduced to <20% compared with total nucleosome monomers³¹) served as the histone source. As the chromatin was not digested with RNase before isolation of nucleosomes, considerable amounts of snRNA presumably complexed in snRNP³² were also present (Chr. Elsner, personal communication).

A suitable DNA fragment containing a tRNA^{Lys} gene was subcloned from cloned nucleosome tetramer DNA¹⁷. The orientation of the fragment with respect to restriction sites of the pBR322 plasmid vector and the position of nucleosomes on the corresponding DNA in chicken embryo chromatin are shown in Fig. 1a. To generate ssDNA stretches of defined length and orientation, a 30-bp fragment of radioactively labelled palindromic DNA was ligated to the gene sequence and the DNA upstream and downstream from the ligation site was differentially digested with exonuclease III. The manipulated DNA was ligated to an acceptor plasmid and the ends of the single-stranded stretches were annealed via the 30-bp palindromic DNA. In the ionic conditions used in subsequent experiments, the annealed DNA (sssDNA) was stable up to 40°C. Oligomerization during ligation reactions was minimized and all recombined fragments had the same orientation within the acceptor plasmid. Moreover, a radioactive label was present at a defined site. The time course of the exonuclease III reaction was monitored by observing the disappearance of restriction sites, when the respective DNA base sequence became single-stranded. Several such sites were spaced in roughly 20-bp intervals or multiples thereof near the 30-bp palindromic DNA. As one restriction site always completely disappeared before the following one began to fade, the variation in length of the single strands at a given incubation time was <20 bp.

For the site-directed assembly process, the soluble nucleoprotein fraction, the histone source and ssDNA were combined to give a highly concentrated solution. The amount of protein provided by the nucleoprotein fraction and the quantity of nucleosomes in the histone source were each equivalent to $\sim 5 \times 10^7$ nuclei. Compared with the total nuclear volume, both constituents were only 10-fold diluted; 10^{-10} mol of nucleosomes and $<5 \times 10^{-13}$ mol of ssDNA were used in a standard assay. During incubation in the presence of the four deoxynucleoside triphosphates and ATP, double-stranded DNA was synthesized from ssDNA and concomitantly nucleosomes were assembled. The positions of the assembled nucleosomes were determined accurately using three complementary methods³³⁻³⁵ (Fig. 1b). The nucleosome position map reveals that the middle axis of one nucleosome always coincided roughly with the midpoint of the ssDNA stretch in the ssDNA used. Single-stranded stretches longer than 120 nucleotides were degraded during incubation and if <40 nucleotides were single-stranded, the assembled nucleosomes associated randomly with respect to DNA base sequence. These data indicate that the stretch of ssDNA itself and/or a protein temporarily bound at about that position direct one nucleosome to a defined site.

tRNA gene transcription within randomly positioned nucleosomes

The soluble ribonucleoprotein fraction of the assembly extract should provide at least 0.02 units per assay of DNA-dependent RNA polymerase III (1 unit incorporates 1 nmol UMP into RNA in 10 min using calf thymus DNA as template³⁰), but no such activity was detected in a complete assembly extract, and partially purified RNA polymerase III was soon inactivated when added to the extract. Therefore, after nucleosome assembly we removed most of the components of the assembly extract by brief ultracentrifugation. This did not yield a pure preparation of assembled nucleosomes but the added RNA polymerase III was now active. The concentrations of protein and DNA in the transcription assay were over one order of magni-

tude lower than in the assembly extract whereas the final concentrations of buffer components were the same. Higher Mg²⁺ concentrations led to precipitation of the assembled nucleosomes, and Mn²⁺ as the divalent cation did not allow for specific transcription. The amounts of RNA polymerase III added provided enzyme saturation.

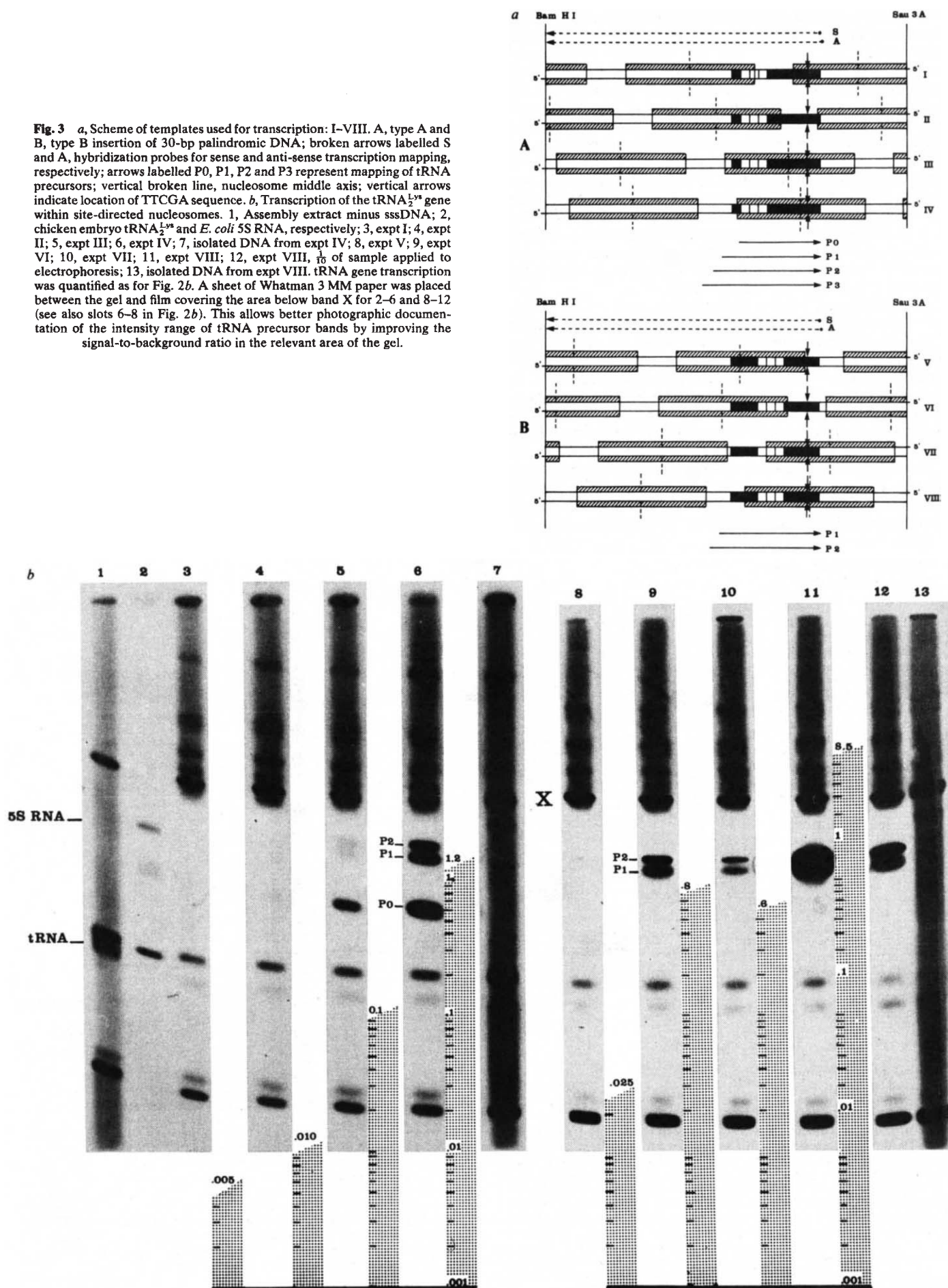
To evaluate transcription experiments, the reference used was transcription of randomly positioned nucleosomes; these were obtained by incubating double-stranded circular DNA with the reconstitution extract. The DNA either had no palindromic insertion or the 30-bp palindromic DNA was inserted into restriction sites for *AluI* (type A insertion) and *HinI* (type B insertion), respectively (Fig. 2a). These templates within randomly positioned nucleosomes were transcribed by the added RNA polymerase III into several discretely sized RNA molecules (Fig. 2b) but there was no apparent mature tRNA synthesized. Nevertheless, several tRNA precursor samples were identified by S₁ nuclease hybridization mapping³⁶ (Fig. 2c).

DNA lacking a palindromic insertion was transcribed to give three kinds of tRNA precursors denoted P1, P2 and P3; these amounted to 22.5% of total RNA synthesis, at a ratio of 3:6:1, respectively. Efficiency of transcription was low, only 1 in 10 tRNA genes being transcribed in 100 min. The precursors each began at the first guanosine residue of the sequence AGGC^A, which is repeated 10 (P1), 21 (P2) and 33 (P3) bp in front of the tRNA structural gene. Assay of randomly positioned nucleosomes containing the type A insertion revealed that P1 and P2 were 30 bp longer, P3 was not detected, and another precursor (P0) was predominant (the ratio P0/P1/P2 was 6:2:2). P0 starts within the tRNA at the 5' end of the 30-bp palindromic insertion which gives in this position the sequence AGCCA; again the guanosine nucleotide is the initiation site. The amount of precursors synthesized was 9% of the total RNA synthesis; ~4% of the tRNA genes were transcribed in 100 min. Type B insertion DNA within randomly positioned nucleosomes was transcribed with the same efficiency as if no insert were present. P0 could not be detected and P2 was again the preferred precursor. Isolated DNA alone of any of the three types was not faithfully transcribed by RNA polymerase III.

tRNA gene transcription depends on nucleosome position

A strikingly different picture was obtained when the tRNA^{Lys} gene was transcribed within site-directed nucleosomes. Four nucleosome positions were assayed which differed with respect to the sequence TTCGA. The four template situations were assembled using sssDNA and type A as well as type B insertions (Fig. 3a). TTCGA was either located near the border of a nucleosome (templates I and V); in the linker DNA (II and VI); ~20 bp from the nucleosome middle axis (III and VII); or very close to the middle axis (IV and VIII). Transcription of tRNA precursors was heavily suppressed in templates I and V (Fig. 3b) although total RNA synthesis corresponded to randomly positioned nucleosomes. Only 0.5% (I) and 2.5% (V) of the tRNA genes were transcribed. In contrast, when TTCGA was located close to the middle axis of a nucleosome (IV and VIII), transcription of tRNA precursors showed dramatic increases of 30-fold for template IV and 85-fold for template VIII, compared with corresponding control experiments using randomly positioned nucleosomes. This is equivalent to one and eight rounds of transcription, respectively, for each tRNA gene. The predominant precursors were P0 for type A insertion DNA (IV) and P2 when type B insertion was used to construct ssDNA. When the nucleosome middle axis was shifted only 20 bp to the right or left of the TTCGA sequence (experiments III and VII) the transcription of tRNA precursors decreased by one order of magnitude (this in turn demonstrates the accuracy of the nucleosome position analysis). Location of TTCGA in the nucleosome linker DNA yielded elevated precursor transcription with the type B insertion only. The amounts of tRNA precursors transcribed were varied over two orders of magnitude by site-directed assembly of nucleosomes. The tRNA^{Lys}

Fig. 3 *a*, Scheme of templates used for transcription: I–VIII. A, type A and B, type B insertion of 30-bp palindromic DNA; broken arrows labelled S and A, hybridization probes for sense and anti-sense transcription mapping, respectively; arrows labelled P0, P1, P2 and P3 represent mapping of tRNA precursors; vertical broken line, nucleosome middle axis; vertical arrows indicate location of TTCGA sequence. *b*, Transcription of the tRNA^{Leu} gene within site-directed nucleosomes. 1, Assembly extract minus sssDNA; 2, chicken embryo tRNA^{Leu} and *E. coli* 5S RNA, respectively; 3, expt I; 4, expt II; 5, expt III; 6, expt IV; 7, isolated DNA from expt IV; 8, expt V; 9, expt VI; 10, expt VII; 11, expt VIII; 12, expt VIII, $\frac{1}{10}$ of sample applied to electrophoresis; 13, isolated DNA from expt VIII. tRNA gene transcription was quantified as for Fig. 2*b*. A sheet of Whatman 3 MM paper was placed between the gel and film covering the area below band X for 2–6 and 8–12 (see also slots 6–8 in Fig. 2*b*). This allows better photographic documentation of the intensity range of tRNA precursor bands by improving the signal-to-background ratio in the relevant area of the gel.



gene investigated was most efficiently transcribed when the sequence TTCGA was associated with the nucleosome middle axis.

To validate these interpretations, we performed two control experiments (Fig. 4). First, after transcription, an aliquot of the transcription assay was routinely digested with restriction endonuclease *Bam*HI. The enzyme should cause cleavage if its site is located in the nucleosome linker DNA whereas cleavage should be suppressed if the site is associated with the nucleosome core (see Fig. 3a). DNA was purified and then restricted with *Sph*I, which cleaved once in the acceptor plasmid downstream from the chicken embryo DNA (see Fig. 1a). Positive *Bam*HI restriction in assembled nucleosomes yielded a radioactively labelled 591-bp fragment after subsequent *Sph*I restriction. In the case of suppressed cleavage, the circular DNA was just linearized by *Sph*I restriction.

Another aliquot of the transcription assay was digested extensively with *Staphylococcus* nuclease to analyse the amount of radioactive label (residing in the 30-bp palindromic DNA) that could be recovered in the nucleosome core DNA. Autoradiographs of the respective fragments separated by agarose gel electrophoresis are shown in Fig. 4a. Obviously, *Bam*HI cleaves template VIII but not template V. The strong band of radioactively labelled nucleosome monomer DNA indicates that in both templates the 30-bp palindromic DNA is associated with the nucleosome core. Taken together, these constitute good evidence that nucleosomes are still in position after transcription. Liquid scintillation counting of the excised agarose gel bands revealed that at least 90% of the nucleosomal

templates used belong to the depicted population (Fig. 3a). If an unrecognized fraction representing <10% of the material, although having a pronounced effect, were the template, the efficiency of transcription would increase to unrealistic values³⁷. We also consider it unlikely that small amounts of ssDNA stretches, persisting after site-directed nucleosome assembly, were the active templates. Templates VI and VII were transcribed with equal efficiency although the coding strand of VI and the noncoding strand of VII, respectively, had to be rendered single-stranded for site-directed nucleosome assembly.

The second control experiment was designed to elucidate further the proteins forming the template. To do this, quantitative amounts of nucleosomes were isolated from the template region. Although several restriction sites seemed suitable to excise the nucleosomal templates by cutting within the respective upstream and downstream nucleosome linker DNAs, only *Bam*HI was active in the transcription assay. Therefore, a subclone was constructed containing an additional *Bam*HI site instead of the downstream *Sau*3A site. Type VIII templates were assembled, incubated for a short period in transcription assays and the excised dimer nucleosomes were purified after *Bam*HI digestion. The yield of protein from 50 such assays (each containing 1 µg of sssDNA) was in the range expected for the dimer nucleosomes (~3–5 µg). SDS-polyacrylamide gel electrophoresis clearly showed (Fig. 4b) that the four nucleosome core histones are the predominantly extracted protein fractions. If other proteins were present in molar concentrations of only half those of the core histones, they would be detected on the

Fig. 4 a, Screening of nucleosome positions after tRNA gene transcription. V and VIII refer to the respective nucleosomal templates (see Fig. 3a). 1, 10 µl of transcription assay restricted with *Bam*HI; 2, 20 µl of transcription assay restricted with *Bam*HI; 3, 10 µl of transcription assay digested with *Staphylococcus* nuclease. Lin, linearized DNA; N1 DNA, nucleosome monomer DNA. For *Bam*HI restriction, aliquots of the transcription assay were diluted to 50 µl with 150 mM NaCl, 10 mM MgCl₂, 5 mM 2-mercaptoethanol, 10 mM Tris-HCl pH 7.9. *Bam*HI (10 U) was added and the suspension incubated at 37 °C for 30 min with brief vortexing every 5 min. Nucleic acids were deproteinized and restricted with *Sph*I. For *Staphylococcus* nuclease digestion, aliquots of the transcription assay were diluted to 50 µl (equivalent to 5×10^7 nuclei ml⁻¹) with 0.25 mM EDTA, 20 mM Tris pH 7.9, and preincubated at 37 °C for 1 min. 1 µl of 50 mM CaCl₂ and 1 µl (1 U) of *Staphylococcus* nuclease were added and incubation continued for 20 min. Nucleic acids were deproteinized and separated on a 1% agarose gel. The gel was dried using DEAE-paper and autoradiographed. Polyacrylamide gel electrophoresis of proteins extracted from the transcription assay and from purified nucleosomal template VIII. 1, Proteins extracted from 15 µl (600 µg protein) of a complete nucleosome assembly extract (25 µl). 2, Total protein extracted from a transcription assay containing a type VIII nucleosomal template. 3, 1.5 µg of the nucleosome monomer preparation (0.7 µg of core histones) serving as the histone source in the site-directed nucleosome assembly system. 4, Total protein extracted from the purified dimer nucleosomes of type VIII nucleosomal templates. Type VIII nucleosomal templates were assembled using sssDNA with a second *Bam*HI site instead of the *Sau*3A site (see Fig. 1 legend). These templates were incubated in transcription assays at 37 °C for 15 min, the MgCl₂ concentration was adjusted to 10 mM, and the suspension digested with 100 U of *Bam*HI at 37 °C for 30 min with brief vortexing every 5 min. Fifty such assays were pooled and dialysed extensively against 20 mM KCl, 1 mM EDTA, 5 mM Tris-HCl pH 7.9. Samples (1 ml) were layered on to 6–30% (w/w) linear sucrose gradients and centrifuged in a SW41 rotor at 150,000g for 15 h. Fractions containing the dimer nucleosomes of interest (identified by the radioactive label residing in the 30-bp palindromic DNA) were recovered, extensively dialysed against H₂O and lyophilized. Proteins were extracted by incubation in 2% (w/v) SDS, 3% (v/v) 2-mercaptoethanol, 0.1% (w/v) phenylmethylsulphonyl fluoride, 10% (v/v) glycerol at 95 °C for 5 min. Proteins were separated on a 5–20% (w/v) polyacrylamide linear gradient gel using as buffer 50 mM sodium phosphate, 0.1% (w/v) SDS. The gel was stained with Coomassie brilliant blue, R-250.

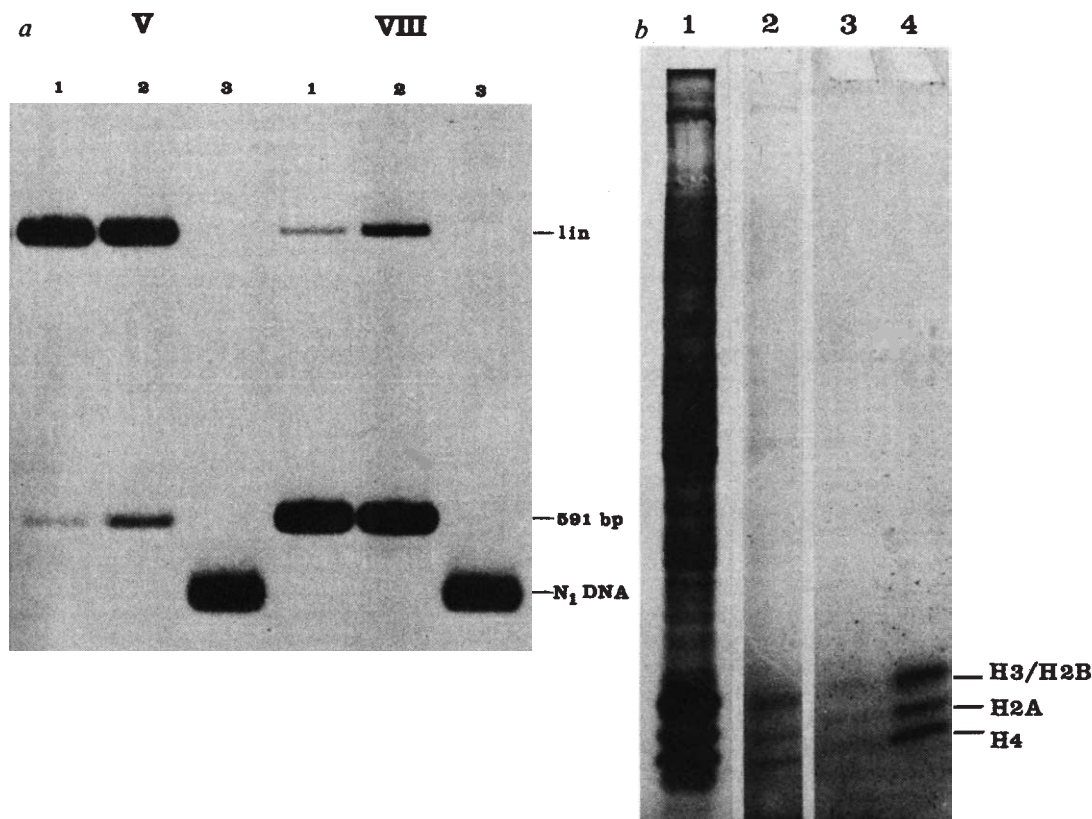
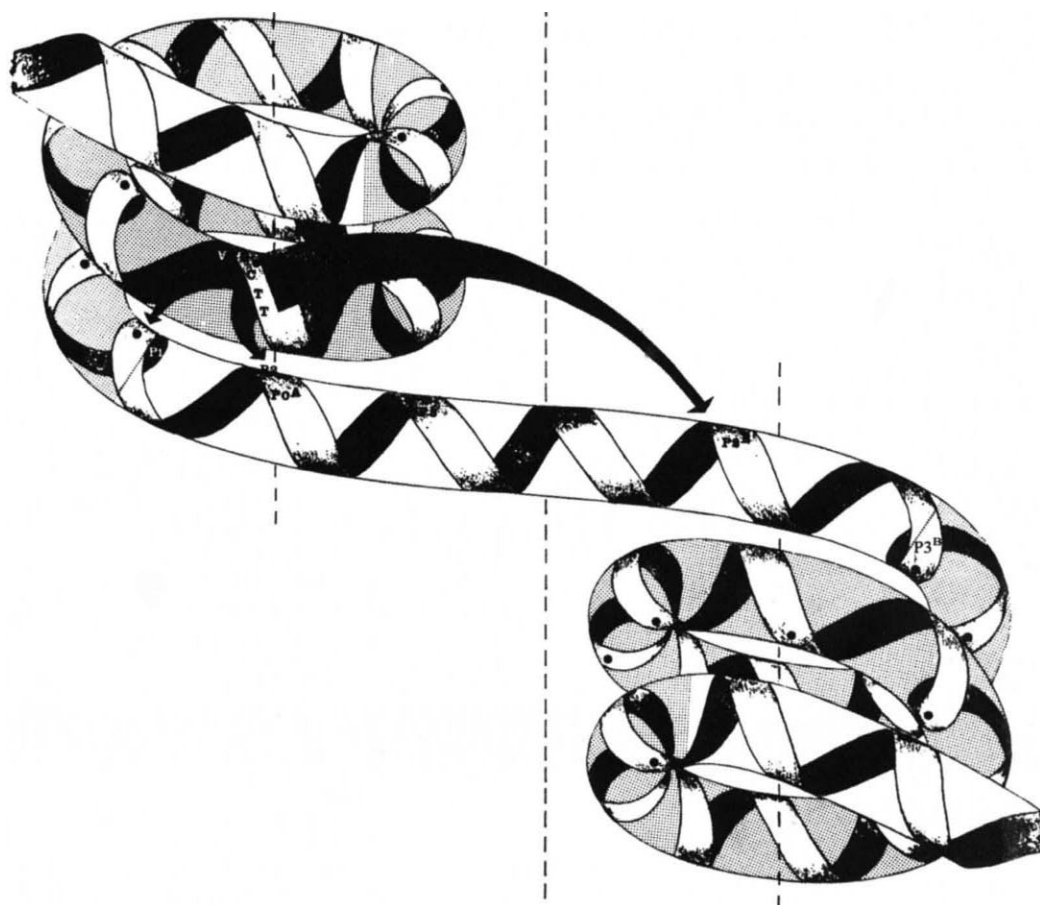


Fig. 5 Functional tRNA gene promoter structure. DNA of the nucleosome core is shown against a shaded background. P1, P2 and P3 represent initiation sites used *in vivo*; P0^A, P1^B and P2^B are initiation sites in assembled nucleosomes containing type A and type B insertion DNA, respectively. P3^B is not used for initiation.



gel. Thus, the protection against nucleases which produced the corresponding nucleosome position maps (Figs 1*b* and 3*a*) could not be effected by proteins other than histones.

Nevertheless, additional proteins are required to promote efficient transcription of the tRNA gene within the assembled nucleosomes. The transcription assay contained a heterogeneous mixture of proteins although it was partially purified from excess reconstitution extract and histone source to allow for RNA polymerase III activity (Fig. 4*b*). However, so far any attempt to purify the nucleosomal template further drastically reduced transcription efficiency. When the RNA polymerase III preparation was added to a finally purified template such as the one used for the protein analysis above, faithful tRNA gene transcription was not observed. We are now investigating which components of the assembly extract and transcription assay are ultimately required using immunological methods.

Conclusions

The construction of sssDNA requires the insertion of a 30-bp palindromic DNA fragment into the tRNA gene. The influence of this insertion on transcription efficiency had to be elucidated before the effect of different nucleosome positions could be interpreted. In this context we have found that insertion of unrelated DNA into the 5'-end region of the tRNA gene (type A insertion) decreases transcription of the gene, whereas the corresponding insertion is tolerated near the middle of the gene (type B insertion). These results are consistent with recent data on consequences of sequence substitutions within a tRNA^{Met} gene²⁵.

The three tRNA^{Lys} precursors observed were not processed to mature tRNA in the *in vitro* system used. We cannot exclude, however, the possibility that precursor P3 alone represents initiation of transcription and that P2 and P1 are equivalents of processing steps³⁸⁻⁴⁰.

The model⁴¹ in Fig. 5 shows the three-dimensional aspects of a functional tRNA gene promoter. The *in vivo* location of the TTCGA sequence within nucleosomes for chicken embryo tRNA genes is identical to the most efficiently transcribed template of nucleosomes assembled *in vitro*. In both cases the TTCGA sequence—the region of symmetry for a 5-bp complementary palindrome—is associated with a region of symmetry of the nucleosome core, that is, the middle axis in two-dimensional schemes. We assume that this structure is primarily recognized by RNA polymerase III, as a deviation from the coincidence of TTCGA with the nucleosome middle axis decreased transcription of the gene. Location of TTCGA near the middle of the nucleosome linker DNA may have an equivalent function (see template VI) although this situation was not detected *in vivo*. The preferred *in vivo* initiation site, P2, and P0^A, the incorrect but preferred initiation site with type A insertion DNA, are located near to the TTCGA sequence. However, the distance to TTCGA is not the main selection principle, as with type B insertion DNA transcription starts predominantly at P2^B and not at the closer P1^B site. The orientation of P2^B with respect to the next nucleosome, which is similar to that of P2 in the first nucleosome, may be the reason for its selection. As we observed no initiation at the hypothesized P3^B site, P2^B may also represent the farthest possible distance from TTCGA. The distance between P1 and P2^B, where initiation sites should principally be located, provides sufficient space to tolerate introns in tRNA genes^{39,40,42}. (In a recent paper¹⁷ a figure shows two cloned tRNA^{Lys} genes containing introns. From the established sequence we now consider these introns to be artefacts because they are duplications of acceptor stem and dihydrouridine stem sequences. They probably originate from the tRNA gene enrichment procedure which involved repair synthesis of double-stranded DNA by *E. coli* DNA polymerase I. Moreover, we were unable to isolate tRNA^{Lys} genes containing introns from a chicken

DNA library (gift of J. D. Engel), although genes corresponding to the one described in the present article were detected.)

We have shown that the functional state of a tRNA gene can be programmed by nucleosome positions. The conserved short DNA base sequence TTCGA which occurs too frequently in the large chicken genome to have any functional significance, is

Received 7 October 1981; accepted 26 March 1982.

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converted into an efficient promoter through a specific arrangement in the nucleosome.

We thank Dr H. Tiedemann for discussion, Chr. Elsner, S. Hense and Chr. Keitel for help in experimentation, and J. Staubach for the graphic design. This work was supported by the Deutsche Forschungsgemeinschaft through Sonderforschungsbereich 29.

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LETTERS TO NATURE

NGC5128—a galaxy with a recently formed disk

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The galaxy NGC5128, better known as the powerful radio source Centaurus A, is a puzzling object appearing as an elliptical galaxy crossed by a dense lane of dust and gas. Its structure and evolution are not well understood, although this object is quite near (we shall adopt 5 Mpc following Burbidge and Burbidge¹), bright, and has been widely observed in the past few years. We have obtained the first complete velocity field of the ionized gas in NGC5128, with a 10-km s⁻¹ precision, from Perot-Fabry interferometry with the ESO 3.6-m telescope. It shows that the gas is rotating in a thick disk (~1 kpc thickness), without any evidence of the perturbations expected from the encounter of an elliptical and a spiral galaxy. Our observations favour the hypothesis of the evolution of a spiral galaxy in which the formation of the disk is very late compared with the bulge.

The velocities in this object were first studied by Burbidge and Burbidge¹ with a slit spectrograph and a 2-m telescope. It was then observed by Sersic² with a 1.5-m telescope, using slit spectrography (mainly for the kinematics of the elliptical component through absorption lines) and Perot-Fabry interferometry (in H α line) for the kinematics of the gas. Eventually Graham³ made a good study of the rotation of the gas and of the motions in the elliptical component with a slit spectrograph attached at the CTIO 4-m telescope.

The more recent photometric study by Dufour *et al.*⁴ gives mainly photometric parameters of the elliptical component; the gas being more difficult to study because of the high inclination

and thickness of the disk, and because of the great quantity of dust.

Our study of the gas in NGC5128 has been made through a narrow bandwidth filter (15 Å FWHM), centred at 6,575 Å, isolating the H α emission line of the ionized gas. Comte and Georgelin took H α plates of the galaxy with a focal reducer and an image tube attached at the ESO 3.6-m telescope. Four interferograms of the object taken with the same device and a Perot-Fabry interferometer (see Table 1) gave interesting information on the morphology of the ionized gas and particularly the kinematics of the gas. They provide the first complete velocity field of the ionized gas in this object.

A preliminary analysis⁵ suggested that the gas is approximately distributed in a disk inclined at ~75° with respect to the line of sight; the temperature of this ionized gas being quite normal as shown by the relatively thin interference lines (<1 Å wide) which indicate no peculiar turbulence⁶.

Such an observation suggests that NGC5128 is not the result of a collision in which a spiral galaxy would have merged into an elliptical galaxy as has been often suggested. The following complete analysis of the velocity field of NGC5128 confirms this opinion.

Another interesting detail is the detection of three bubble-like H II regions⁵ of ~200 pc in diameter (assuming that the galaxy is at 5 Mpc). This is quite comparable with bubble regions observed in nearby spiral galaxies such as M31 (ref. 7), M33 (ref. 8) or NGC925 (ref. 9).

Concerning the morphology of the galaxy itself, we obtained a striking image by viewing simultaneously on a screen a H α plate (projected through a red filter) and a visible plate (projected through a green filter). The two components of the galaxy are quite distinct: the old elliptical component appears as a yellow ball, while the surrounding gas and dust show up vividly in red and black. The plates used for this composite image are I 1864 (40-m in exposure) and I 1886 (30 s exposure), see Table 1. Due to our very narrow H α interference filter (15 Å FWHM) and our fast aperture ratio, $f/2$ with a 3.6-m telescope, the H II regions are much clearer than on the colour photograph obtained by Dufour *et al.*^{4,10} with a larger H α filter (54 Å FWHM) but with a slow aperture ratio, $f/10$ with a 1-m telescope. This is especially true a long way from the nucleus where

Table 1 Plate material

Plate no.	Date	Exposure (min)	Emulsion	Filter	Focal reducer	Perot-Fabry (interference order)	Quality	No. of velocity points per interferogram
I 1864	12.02.78	10*, 40*	IIIaJ (B), N ₂	H α (15 Å)	<i>f</i> : 1.9	No	Very good	
I 1881	4.04.78	150	098.02 (B), N ₂	H α (15 Å)	<i>f</i> : 0.95	1051	Good	135
I 1886	5.04.78	0.2*, 0.5*, 1.5*	IIIaJ (B), N ₂	Orange (wide)	<i>f</i> : 1.9	No	Good	
I 1887	5.04.78	90*	IIIaJ (B), N ₂	H α (15 Å)	<i>f</i> : 1.9	1051	Very good	288
I 1888	5.04.78	60*	IIIaJ (B), N ₂	H α (15 Å)	<i>f</i> : 1.9	1051	Good	241
I 1889	5.04.78	60*	IIIaJ (B), N ₂	H α (15 Å)	<i>f</i> : 1.9	1051	Good	214

* With RCA image intensifier.

one can see nothing but dust on their photograph (image tubes were used by both teams).

The measurement of the four interferograms gave about 900 velocity points which were averaged in 210 squares of 15 arc s a side on the sky (see Fig. 1). The aim of averaging the velocity points is to reduce the internal dispersion of the measurements and to facilitate the drawing of the isovelocity lines. In each square we computed the dispersion of the measurement (not taking into account the dispersion inside each single interferogram), which gives an idea of the expected precision for the value of the radial velocity. We then computed the statistical error, for each square, defined as $s/\sqrt{n}$ where n is the number of interferograms in the considered square. The average of the statistical error indicates a mean precision of 11 km s⁻¹ on the whole velocity field. This allows us to draw the isovelocity lines with a 30 km s⁻¹ step.

The general design of the velocity field (see Fig. 2) is quite comparable with the velocity field of spiral galaxies such as M83 (NGC5236) (ref. 11), NGC7793 (ref. 12), or NGC253 (ref. 13) (although there is evidence for non-axisymmetric motions in the central parts of this galaxy, possibly due to a

weak bar). This supports the idea that the ionized gas is laying in a disk, as already suggested from the elliptical shape of the distribution of the H II regions³ and from the colour analysis⁴. The superimposition of all of our interference fringes on the same picture also indicates that the disk is inclined to roughly 75° with respect to the line of sight. This last method is very powerful for the H α detection because of the high contrast it offers¹⁴, about 1,000 times the contrast of a 250 Å wide colour filter. Nevertheless, both morphological and kinematical points of view rule out the possibility of a cylindrical structure as suggested by Rodgers and Harding¹⁵. Furthermore, there is no evidence of strong non-circular motions in our velocity field. However, near the minor axis the isovelocity lines are not exactly perpendicular to the major axis, their inclination suggesting some accretion (if one considers that the northern side of the disk is nearer than the southern one, as clearly shown by the dust lane appearance).

The most noticeable perturbation in our velocity field is at ~1 arc min west of the nucleus and can be explained by a strong thickness of the gaseous disk which would be mainly concentrated in a thick belt surrounding the elliptical component. The

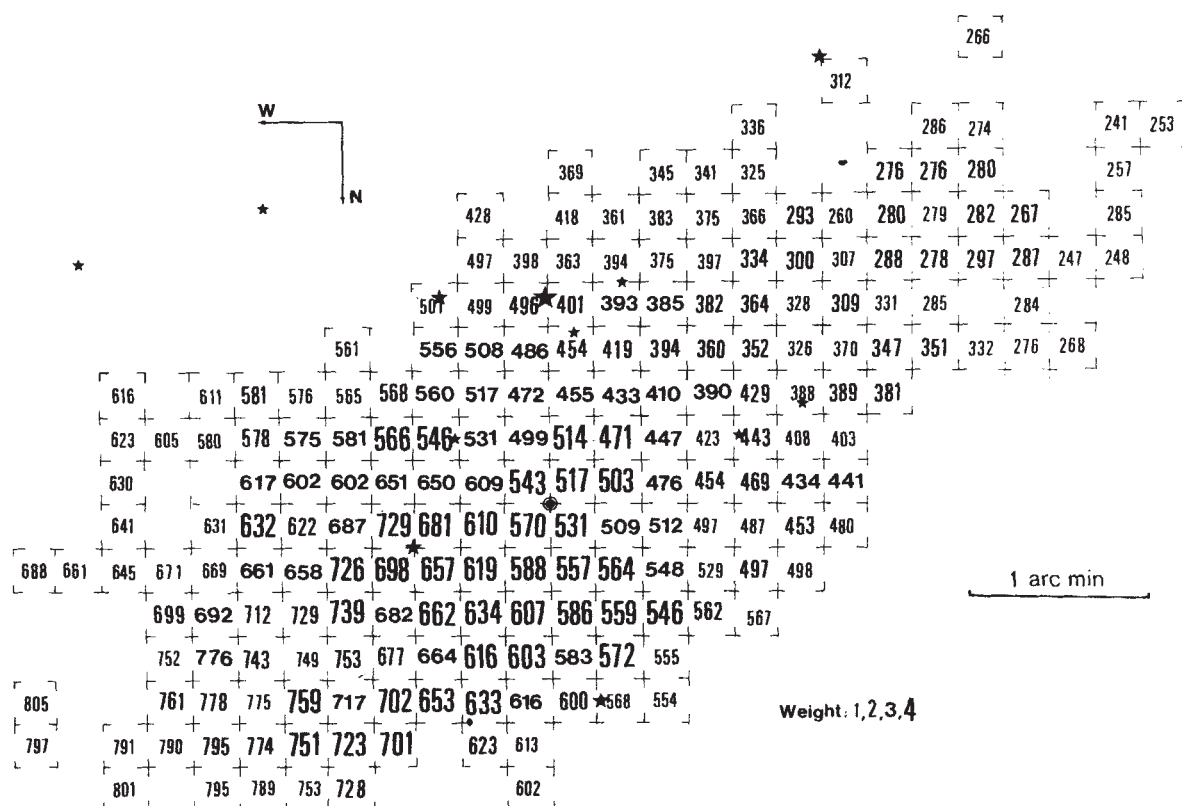


Fig. 1 Velocity measurements in NGC5128 averaged in squares of 15 arc s side. Four different sizes of figures are used, depending on how many interferograms (1, 2, 3 or 4) are averaged in a given square. The position of the nucleus is indicated (dotted circle) also with reference stars.

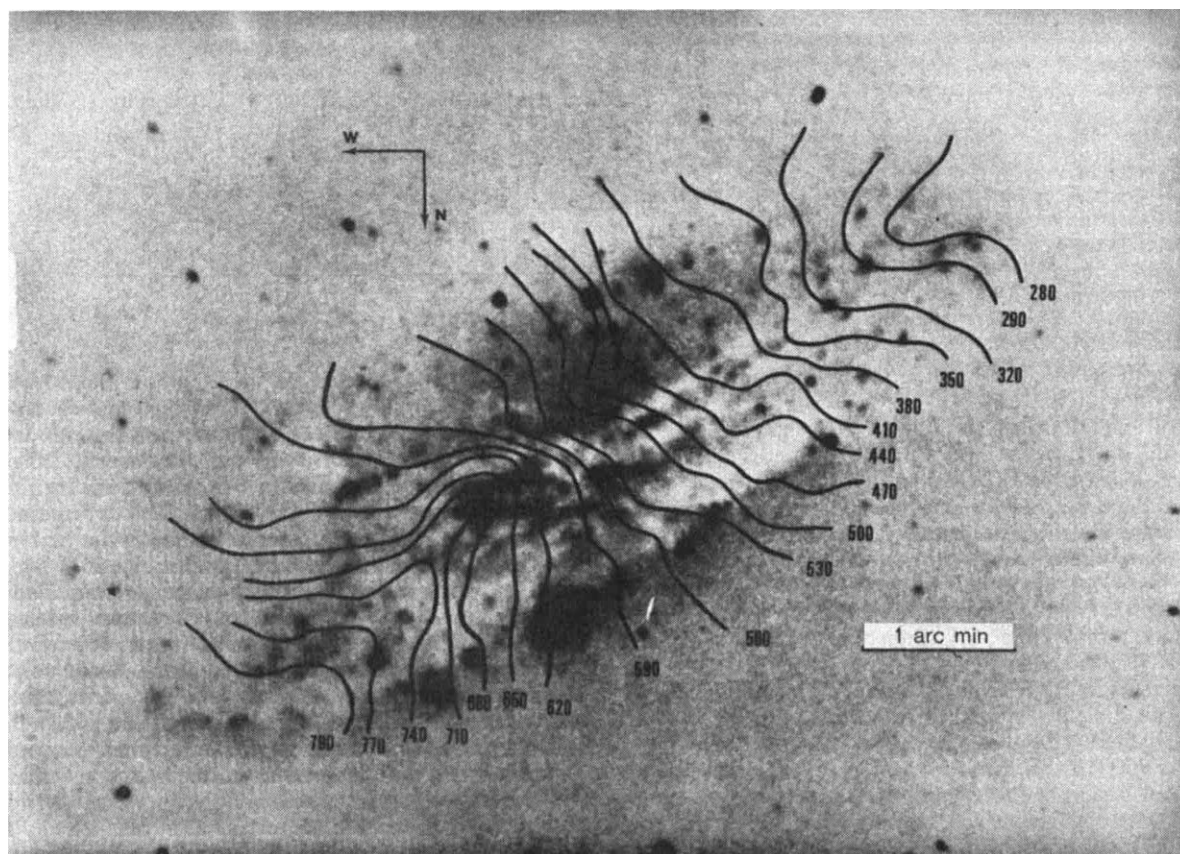


Fig. 2 Isovelocity lines superimposed on a $H\alpha$ photograph of NGC 5128. These lines are eye-estimates from Fig. 1, taking into account the weight of the data.

location of the maximum velocity gradient in this zone indicates that we are looking at gas ~ 1 kpc above the mean plane of the disk, this gas masking the underlying disk. Such an extension of matter out of the mean galactic plane is confirmed by the morphology of the galaxy where one can see dust high above this plane silhouetted on the elliptical component. This would also explain the velocity anomalies stressed by Möllenhoff¹⁶. However, we could not confirm the superposition on the line of sight of objects with different radial velocities mentioned by some observers^{9,15}.

Another interesting detail is the straight chain of dust and H II regions stretching along 2 arc min from the nucleus towards the south-east. This chain also seems to be out of the mean plane (because it is silhouetted in front of the elliptical component while the nucleus is seen slightly under it when looking at the disk from above, with north down) and may be connected to the western part mentioned above. When studying the rotation curve of the disk, the assumption that it is flat would diminish the velocity gradient in the central part of the galaxy because the gas along the major axis is masked by gas above the plane. This gas being out of the plane of the disk, and away from the major axis, indicates slower radial velocities than expected on this line of sight and hence apparently slower rotation velocities. This remark will be helpful when analysing the rotation curve in the first 2 arc min from the nucleus.

Taking into account the squares lying within 33° (in the sky) of the major axis we obtain the rotation curve of Fig. 3 where we also plotted Graham's points³. The agreement is fairly good between the two sets of data. The parameters giving the least dispersion for our points are close to Graham's parameters³. From these data we have found: inclination of the plane of the galaxy with respect to the plane of the sky, $i = 73^\circ \pm 3^\circ$; position angle (PA) of major axis = $127^\circ \pm 5^\circ$; systemic velocity $V_s =$

$545 \text{ km s}^{-1} \pm 5 \text{ km s}^{-1}$ (while Graham³ found, respectively, $i = 73^\circ$, PA = 125° and $V_s = 548 \text{ km s}^{-1}$).

These last values of inclination and position angle of the major axis agree well with previous works^{1,2,4,16}. The main problem concerns the systemic velocity for which Burbidge and Burbidge¹ found 605 km s^{-1} , Sersic² 462 km s^{-1} , Kunkel and Bradt¹⁷ 508 km s^{-1} , Möllenhoff¹⁶ 602 km s^{-1} . Our result, 545 km s^{-1} , is in very good agreement with the most recent papers of Graham³, 548 km s^{-1} , or Whiteoak and Gardner¹⁸ who find 551 km s^{-1} from H I observations.

Up to 4 arc min from the nucleus the symmetry of the two quadrants of the rotation curve is pretty good. Beyond 4 arc min we have almost no information for the southeastern quadrant, although we observe (somewhat lessened) the same tendency as Graham. That is, we see an increase of the velocity gradient in the outer part of the disk, which is probably due to matter moving out of the mean plane. Between 4 and 7 arc min in the northwestern quadrant the velocity gradient seems, in contrast, to diminish rapidly. Despite the small number of points available there, it seems probable that the rotation curve reaches a maximum between 5 and 7 arc min. The dashed line of Fig. 3 indicates a maximum rotation velocity $V_M = 352 \text{ km s}^{-1}$ at $r = 5.35$ arc min. This symmetric curve is a least square fitting polynomial (of the third degree) obtained from our points within 20° of the major axis to which we added the two outermost points given by Graham³, also with our outermost point (this last point is in fact at 52° from the major axis but it is in very good agreement with Graham's outermost points³). Although their weight is rather poor and they may be out of the mean plane of the disk, it is the only way of extending our rotation curve beyond 4 arc min.

Following the method of Burbidge *et al.*¹⁹ this least square curve indicates a mass $M = 0.9 \times 10^{11} M_\odot$ to $1.0 \times 10^{11} M_\odot$.

within 4 arc min and a mass $M = 1.8 \times 10^{11} M_{\odot}$ to $2.0 \times 10^{11} M_{\odot}$ within 7 arc min, depending on the eccentricity of the ellipsoids used in the model. This value is smaller than the rough estimate $3 \times 10^{11} M_{\odot}$ given by Graham³ from Kepler's law applied to the outermost points.

We have tried a more sophisticated method developed by Monnet and Simien²⁰ in which a model galaxy is built up from two components: an elliptical bulge and a flattened disk. This model shows that the bulge generally gives rise to a very steep velocity gradient near the nucleus, while our observed rotation curve surprisingly looks like a pure disk rotation curve, thus suggesting at once that we are dealing with a massive disk and a rather light bulge. The photometric parameters used for the spherical bulge were those given by Dufour *et al.*⁴ (equivalent radius $r_e = 305$ arc s with a surface brightness $\sigma_B(r_e) = 22.94$ mag arc s⁻², which gives a luminosity $I_e = 44.1 \mathcal{L}_{\odot} \text{pc}^{-2}$). For the disk we used the photometric parameters given by Freeman²¹ from Sersic's isophotes ($r_e = 336$ arc s, $I_e = 34 \mathcal{L}_{\odot} \text{pc}^{-2}$), although, because of the great quantity of dust and because of the high inclination these parameters are not very reliable.

Assuming a constant $M/L_B = 5$ for the bulge, which is the value expected for such an elliptical component^{20,22} we find a theoretical rotation curve for the bulge alone where the gradient near the nucleus is five times the observed gradient, with a maximum of more than 300 km s^{-1} near 1.5 arc min, before slowly decreasing. Comparison with Fig. 3 shows that the discrepancy between this model and the observations is much too high, even if we take into account the projection effect mentioned above, which would lessen the gradient in the inner part of the curve. Values of M/L_B between 1 and 3 for the bulge, comparable to what is found in globular clusters²³, are far more acceptable when comparing the model with the observations.

Eventually we found a satisfactory fit with an average $M/L_B \sim 2$ for the bulge and $M/L_B \sim 30$ for the disk, which is rather high but seems acceptable for a spiral galaxy²⁰. From the total luminosity of each component we thus deduce a total mass of $1.1 \times 10^{11} M_{\odot}$ for the bulge, $8.2 \times 10^{11} M_{\odot}$ for the disk and $9.3 \times 10^{11} M_{\odot}$ for the galaxy as a whole. An intermediate solution with a varying M/L_B for the bulge (from 1 at the centre, up to 5 around 5 arc min) and a constant $M/L_B \sim 15$ for the disk could give a better fit, but it is not very realistic to assume a varying M/L_B for such a homogeneous stellar population. The mass would be then around $2 \times 10^{11} M_{\odot}$ for the bulge and $4 \times 10^{11} M_{\odot}$ for the disk, thus giving a total mass around $6 \times 10^{11} M_{\odot}$. The result remains much greater than our first estimate, based on the rotation curve, mainly because it relies on

the outermost photometric data and hence takes into account a much greater part of the galaxy down to the sky background.

Note that, in any case, the disk component is found to be more massive than the elliptical component, while the bulge is much more luminous.

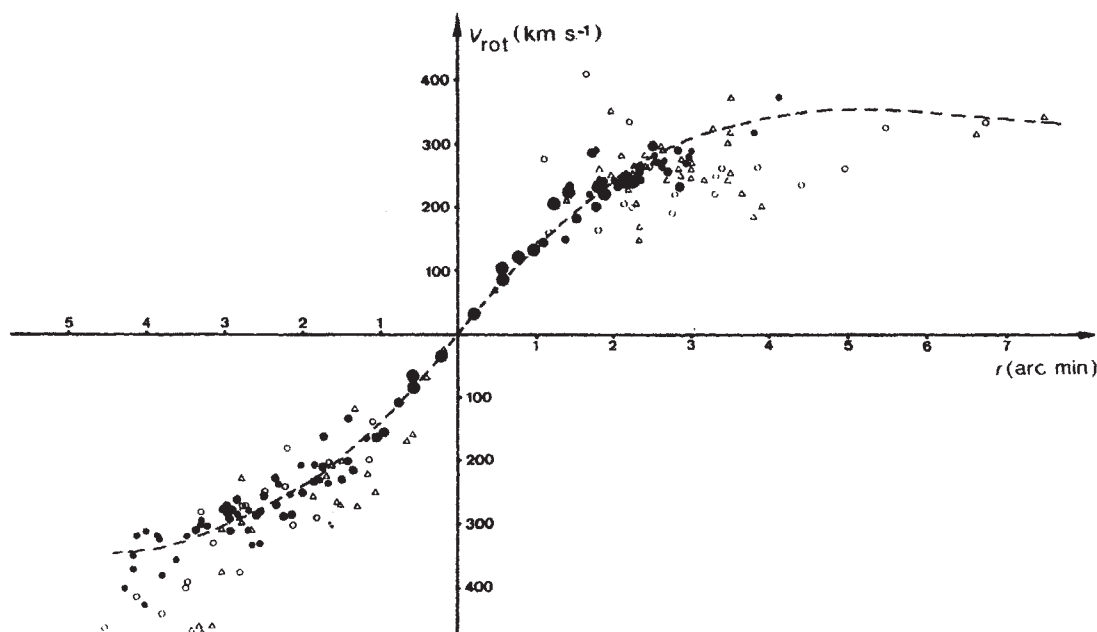
We will now try to review briefly some of the different hypotheses concerning the possible evolution of NGC5128.

(1) The encounter of an elliptical and a spiral galaxy is strongly suggested by the two clearly different populations (an old stellar population in a bright elliptical bulge and a young stellar population, rich in gas and dust, in a disk). The main problem is that there is no evidence for the encounter, such as strong erratic motions in the gas. Furthermore, there is no significant difference between the systemic velocity we found for the disk, $V_s = 545 \text{ km s}^{-1} \pm 5 \text{ km s}^{-1}$, and the velocity found by Graham³ for the elliptical component, $536 \text{ km s}^{-1} \pm 30 \text{ km s}^{-1}$. Also, there is no trace of the nucleus supposed to have belonged to the spiral galaxy as Dufour *et al.*⁴ emphasize. Then it is surprising that the main axis of the slightly prolate bulge ($PA = 35^\circ$)⁴ and the rotation axis of the disk ($PA = 37^\circ$) are the same, within 2° . At least there are many other comparable galaxies having a prolate spheroid as a bulge, which suggest that they are a peculiar type of object rather than the result of an encounter. For example: NGC5363, NGC1947, Cyg A and PKS1934-63 (ref. 24), NGC2865 (ref. 25) (the Spindle galaxy) and NGC612 (ref. 26) which is a radiogalaxy quite similar to NGC5128 in both radio and optical wavelengths.

(2) There are fewer objections to accretion of a gas cloud by an elliptical galaxy than there are for the above hypothesis. Furthermore, as already mentioned by Graham, the huge dust lane on the eastern side of NGC5128 seems to indicate the direction of accretion of matter coming from outside of the disk plane. However, the accretion of a primeval gas cloud can hardly explain why there is so much dust and why the H II regions are as metal rich as normal H II regions of spiral galaxies⁴. Furthermore, this hypothesis would imply a peculiarly favourable accretion angle to make the main axis of the disk and bulge coincide as well. An interesting model of accretion, studied by Tubbs²⁷, supposes that the mass of the disk is negligible, which is far from being the case as we have seen above.

(3) We prefer the hypothesis of a normal spiral galaxy (with late formation of the disk) of SO or Sa type, composed of a bright bulge and a thick disk. This disk is still under formation, undergoing accretion of dust and gas and slowly settling down. Larson's theory²⁸ on the evolution of elliptical and spiral galaxies could help explaining such an object if one assumes

Fig. 3 Rotation curve of NGC5128. ●, Our velocity points within 20° from major axis which have different sizes, depending on their weight on Fig. 1; ○, our velocity points between 20° and 33° from major axis; △, Graham's velocity points. The dashed line is a least square fitting curve (see text).



that the protogalactic cloud was very large with a high density at its centre.

In the Larson's hypothesis²⁸ of a two-phase structure the dense clouds will rapidly form stars in a spheroidal component (which may be prolate as showed by Gott and Thuan²⁹), while the less dense intercloud gas will take a long time to settle to a disk and form stars. The continuing formation of this disk could explain the activity of NGC5128 and the associated powerful radio source.

Received 1 February; accepted 6 April 1982.

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Field stimulated exoelectron emission from borosilicate glass

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Exoelectrons, those electrons in energy levels of solids which are not fed by normal conduction mechanisms, have been detected at the surface of various materials¹. Emission of exoelectrons may occur during mechanical stress², chemical reaction³, or on heating⁴ or irradiation by photons of suitable energy⁵. Thermally stimulated exoelectron emission gives rise to so-called glow curves, in which the emitted current goes through a maximum as the temperature is increased. Robertson¹ has suggested that an electric field may stimulate exoelectron emission in an analogous manner to thermal and photo emission. We describe here experiments which demonstrate the phenomenon of field stimulated exoelectron emission (FSEE), and discuss its relation to steady-state emission.

Electron emission from borosilicate glass under the influence of a strong electric field was first demonstrated by Hibbert and Robertson^{6,7}. The emission current was continuous, noisy, of low intensity, and occurred from discrete 'active sites' on the glass surface. Further work⁸ revealed that the total current is made up, in part, of fluctuating bursts from the active sites, whose characteristics are similar to each other. We have observed that on applying a high voltage to the glass emitter, a pulse of electrons is emitted, after which the steady-state emission is recorded. We suggest that the initial pulse of electrons is due to FSEE.

The apparatus (Fig. 1) for detecting FSEE was an evacuated (10^{-4} Pa) chamber containing a clean (trichloroethylene, chromic acid, distilled water), cylindrical Pyrex glass emitter surrounded by an earthed nickel gauze having 80 spaces per

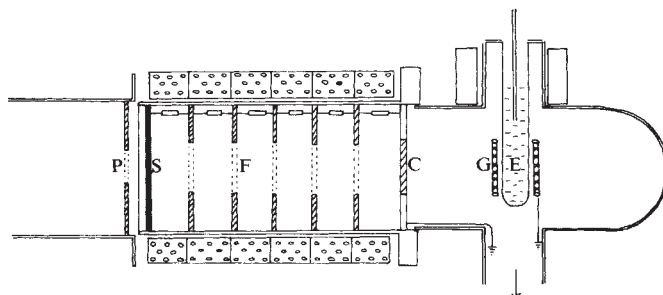


Fig. 1 Apparatus for detecting FSEE. E, emitter; G, nickel gauze; C, channel plate; F, focusing electrodes; S, phosphor screen; P, photomultiplier.

inch. The field at the surface of the glass for an applied voltage V is:

$$E = V\epsilon_1/[r_1\epsilon_2 \ln(r_1/r_0) + r_1\epsilon_1 \ln(r_2/r_1)]$$

where r_0 , r_1 and r_2 are the internal and external radii of the glass tube, and the radius of the nickel gauze respectively. ϵ_1 and ϵ_2 are the relative permittivities of glass and a vacuum ($\epsilon_1 = 4.8$). For $r_0 = 5.5$ mm, $r_1 = 6.5$ mm and $r_2 = 9.5$ mm, $E = 370$ V Vm⁻¹. The range of voltages applied in these experiments was 3–15 kV. Electrons which passed through the gauze were detected by a channel plate electron multiplier (Mullard

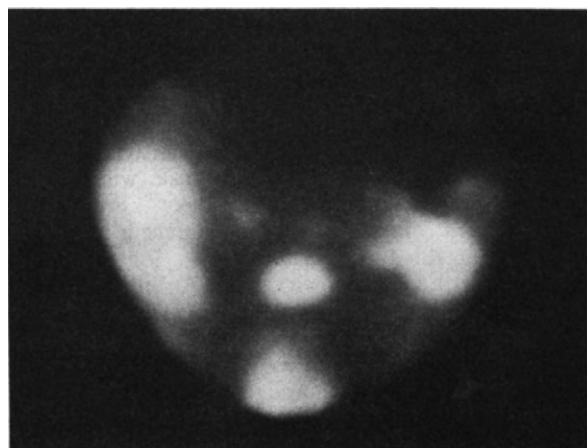


Fig. 2 Emission pattern of 13 mm diameter glass tube showing 2.5 mm diameter central area. Field at surface 2.59 MV m⁻¹.

G40/25) and a pattern of the emission from a section of the glass surface was displayed on a phosphor screen. A photomultiplier detected the light output from the screen. By suitable masking and focusing, the emission from a single active site was recorded. The photomultiplier output was calibrated and found to be proportional to the emission current in the range studied. Steady-state emission followed a Schottky-like equation⁶:

$$I = N \exp [-(\phi - aE^{1/2})/kT]$$

where N and a are constants (experimentally $a = 1.8 \times 10^{-4}$ eV V^{-1/2} m^{1/2}), k is Boltzmann's constant, E the electric field strength, ϕ ($= 0.97$ eV) the energy difference between the electron levels and the vacuum level in the absence of an electric field, and T the absolute temperature. Exoelectron emission was studied by applying a voltage step to the glass emitter, and following the time development of the emission current on an oscilloscope. In addition, a ramped voltage was applied in an attempt to produce a glow curve of exoelectrons.

Figure 2 shows the steady-state emission pattern at 7 kV from a section of the borosilicate glass emitter. On stepping from 0 kV to the operating voltage each site showed a pulse of brightness which was recorded on the oscilloscope (Fig. 3).

The height of the current pulse was dependent on the recovery time from switching off the previous voltage step. For a recovery time of <100 ms no exoelectron emission could be detected; ~ 10 s was required for the pulse to be restored to its maximum height (Fig. 4). The height of the pulse increased with both applied voltage and temperature. Such pulses were detected at the lowest voltage of 3 kV. A ramped voltage (0 V to 10 kV at 300 V s^{-1}) gave no glow curve. We attribute this to the ease at which exoelectrons are emitted by electric fields and the existence of a steady-state emission current which would mask any glow curve.

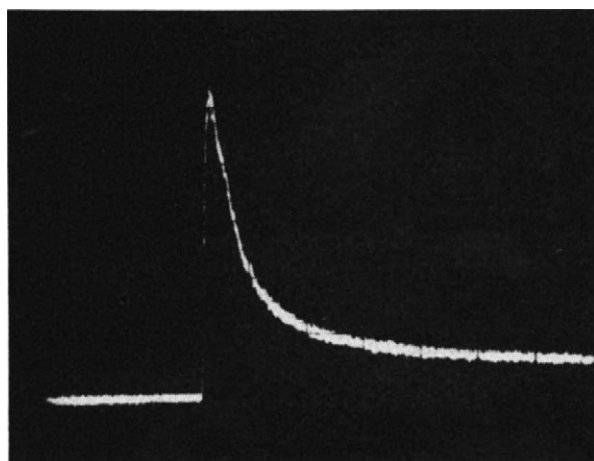


Fig. 3 Oscilloscope trace of the photomultiplier output of a burst of electrons from a single site, on stepping the emitter voltage from 0 V to 15 kV; 1 division on time axis = 0.5 s.

The connection between the exo and steady-state emission is still to be fully determined. Although steady-state emission may come from discrete energy levels in borosilicate glass⁶ (there being no recognized conduction electrons), because of the slow, activated replenishment of exoelectrons, it is unlikely that the two types of emission occur from the same levels. Exoelectron emission has also been shown to depend strongly on surface conditions, and may disappear entirely in ultra-high vacuum⁹. Steady-state emission from glass is, however, maintained in ultra-high vacuum⁷.

In conclusion, the pulse of electrons observed on applying a high voltage appears to be of exoelectrons; they are weakly

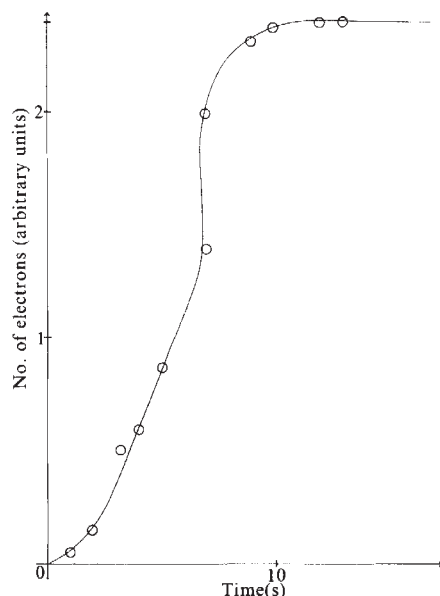


Fig. 4 Electron burst, measured as the height of the oscilloscope trace, as a function of the recovery time before a voltage step of 10 kV.

held at the surface, and are only slowly replenished after emission. It should now be possible to investigate exoelectrons from other materials by the voltage step method. In particular, metals used as catalysts may be studied by this technique.

Received 8 February; accepted 12 March 1982.

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Helium isotopic systematics of oceanic islands and mantle heterogeneity

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Isotopic variations in oceanic igneous rocks provide important constraints on models of oceanic mantle structure. Of particular interest is the global negative correlation between $^{87}\text{Sr}/^{86}\text{Sr}$ and $^{143}\text{Nd}/^{144}\text{Nd}$, which has been used to estimate 'bulk earth' values^{1–3} for $^{87}\text{Sr}/^{86}\text{Sr}$, $^{87}\text{Rb}/^{88}\text{Sr}$ and $^{143}\text{Nd}/^{144}\text{Nd}$. Simple two-reservoir models have failed to explain all the isotopic variations, however, because of the complicated trends in Pb isotopes^{4–6}. This has led to suggestions that recycled oceanic crust or sediments must be considered in these models^{7–9}. We report here the results of helium isotopic analyses in basaltic phenocrysts from the islands of Gough and Tristan da Cunha. Because basalts from the islands lie near bulk earth on the Sr–Nd correlation diagram³, the study was initiated to characterize the helium isotopic signature of this component. Whereas the ^3He in mantle gases is mostly primordial, the ^4He is primarily radiogenic, having been produced by decay of ^{238}U , ^{235}U and ^{232}Th . High $^3\text{He}/^4\text{He}$ ratios in igneous rocks therefore indicate primordial volatiles^{10,11}. We believe that the present results are inconsistent with the notion that the mantle beneath Gough and Tristan da Cunha is primitive or undepleted relative to mid-ocean ridge basalt (MORB). Helium isotopic results on basaltic glasses and phenocrysts from the rift zone of Kilauea confirm the previously reported high values from this area^{12–15}. We also report new analyses from Loihi Seamount (40 km south-east of Kilauea), which does seem to be derived from a more primitive source. When these data are combined with values for MORBs (from ref. 16) and plotted with respect to $^{87}\text{Sr}/^{86}\text{Sr}$, the observed trends offer insight into the different source regions for oceanic island basalts and the nature of mantle heterogeneity.

As sub-aerial basalts are largely degassed, they are of little use in the analysis of magmatic gas. This contrasts with MORB glasses, which are quenched rapidly enough on the ocean floor to trap substantial quantities of the magmatic gas^{17,18}. Kaneoka *et al.*¹², however, have shown that the olivine phenocrysts from Kilauea basalts trap some of the magmatic gases that existed during crystal growth. The present results confirm the high $^3\text{He}/^4\text{He}$ ratios they reported for Kilauea phenocrysts, and we extend the approach to basalts from several other islands.

The samples were lightly crushed in a steel mortar, sieved, and the phenocrysts in the 20–40 mesh size range were separated by 'Frantz isodynamic' separation and hand-picking. Glassy samples were handpicked to exclude alteration, oxide

crusts and non-vitreous chunks. Because the vesicles in submarine basalts can be a source of gas loss, chips >2 mm in size were selected¹⁹. After sonic cleaning, 1–2 g of the phenocrysts, or 400–500 mg of glass, were placed in a stainless-steel vessel designed specifically for *in vacuo* crushing¹⁹. The details of the gas mass spectrometry are similar to those reported earlier¹⁹, except that the released helium was purified and expanded directly into the mass spectrometer, which resulted in a lower procedural blank ($1.0\text{--}2.0 \pm 0.3 \times 10^{-9} \text{ cm}^3 \text{ STP } ^4\text{He}$ with atmospheric $^3\text{He}/^4\text{He}$). Due to the small sample size, variability in the blank is the primary contributor to the experimental uncertainty (see Table 1).

Strontium isotopic analyses of several of the samples were performed using techniques that have been described elsewhere²⁰. The measured $^{87}\text{Sr}/^{86}\text{Sr}$ ratios for Loihi Seamount samples KK 20-4 and KK 23-3 were both 0.70358 ± 0.00002 , and Staudigel *et al.*²¹ reported a value of 0.70358 ± 0.00004 for sample KK18-8. Gough Island samples ALR 26G and ALR 41G had $^{87}\text{Sr}/^{86}\text{Sr}$ ratios of 0.70527 ± 0.00004 and 0.70521 ± 0.00005 respectively; the Prince Edward sample WJ21E had a ratio of 0.70305 ± 0.00004 . These values are reported relative to an Eimer and Amend standard value of 0.70800; the errors are 2σ for in-run statistics.

The helium results are reported in Table 1 for phenocryst samples from Tristan da Cunha, Gough, Prince Edward, Jan Mayen and Kilauea. We also report the analyses of submarine basaltic glass from the Loihi Seamount and the east rift of Kilauea, and the rock type and sample source for each sample are listed; a more detailed description is given in ref. 22.

Examination of the phenocryst samples in thin section shows that the most likely residence site for the helium is in the ubiquitous melt inclusions. In most cases these inclusions have undergone some post-entrapment crystallization, which explains why most of the helium is released by crushing *in vacuo* (see Table 1). Crystallization excludes the gas from the melt, but helium is still trapped in the phenocryst. The absence

of xenocrysts in these samples was verified by petrographical examination²².

In testing whether phenocrysts can be used for gas analysis, the samples from Kilauea were chosen because they are well characterized, and because they allow comparison of the phenocryst helium with the magmatic helium. The good agreement between the phenocrysts from the Kilauea picrite (H66050) and the two submarine glasses from the same volcanic rift supports the use of phenocrysts to indicate magmatic $^3\text{He}/^4\text{He}$ ratios. Our results also confirm the relatively high ratios in Kilauea phenocrysts reported by Kaneoka *et al.*¹², and by several laboratories for the Kilauea fumaroles^{13,14}. The Loihi samples have even higher $^3\text{He}/^4\text{He}$ ratios, up to 31.9 times atmospheric, but have $^{87}\text{Sr}/^{86}\text{Sr}$ ratios that are indistinguishable from the values for the Kilauea samples²³. Note that Kaneoka and Takaoka¹⁵ have reported even higher $^3\text{He}/^4\text{He}$ ratios (up to 37 times atmospheric) for phenocrysts from Haleakala (Maui).

In contrast, the Tristan da Cunha and Gough samples contain helium with $^3\text{He}/^4\text{He}$ ratios significantly lower than the MORB values. As it was impossible to separate the amphibole in TK 26 from the interstitial opaque oxides and other accessory minerals, and because there is some question about the origin of these gabbroic nodules²⁴, we also analysed a basalt from Tristan da Cunha that contained large phenocrysts (TK 46A). Special care was taken to avoid opaque oxides and possible host rock contaminants (U and Th rich phases). The similar $^3\text{He}/^4\text{He}$ ratio for two different phenocryst phases from the same sample (TK 46A and ALR 26G) and between different volcanic eruptions suggests that this is not a problem.

While Kilauea, Loihi Seamount, Tristan da Cunha and Gough all have $^{87}\text{Sr}/^{86}\text{Sr}$ ratios higher (more radiogenic) than MORB values, the Hawaiian samples have higher $^3\text{He}/^4\text{He}$ ratios and Tristan and Gough have lower $^3\text{He}/^4\text{He}$ ratios. As shown in the plot of $^3\text{He}/^4\text{He}$ against $^{87}\text{Sr}/^{86}\text{Sr}$ (Fig. 1), the results fall into two distinct trends. For reference, we also show MORB

Table 1 Helium isotopic analyses for phenocryst and glass samples

Sample	Phase analysed	Rock type and location	^4He $\text{cm}^3 \text{ STP g}^{-1}$	σ	$^3\text{He}/^4\text{He}$ (R/R_a)	σ	Sample source
Tristan da Cunha							
TK 26	Amphibole	{ Gabbro xenolith in pyroclastics, Buff Gulch	3.3×10^{-7}	0.1	5.2	0.1	†
*TK 26	Amphibole		1.0×10^{-7}	0.1	4.7	0.1	†
TK 46A	Olivine	{ Ankaramite, near Big Point	7.3×10^{-9}	0.8	6.3	0.7	†
*TK 46A	Olivine		1.0×10^{-9}	0.1	5.6	2.1	†
TK 46A	Clinopyroxene		3.5×10^{-8}	0.2	5.1	0.3	†
Gough Island							
ALR 41G	Clinopyroxene	{ Highly pyroxene phyric basalt, Mount Powett	1.3×10^{-9}	0.4	4.9	1.6	‡
*ALR 41G	Clinopyroxene		$< 7.0 \times 10^{-10}$	—	—	—	‡
ALR 41G	Clinopyroxene	{ Ankaramite, Mount Powett	5.8×10^{-10}	0.9	5.5	0.7	‡
ALR 26G	Olivine		1.53×10^{-8}	0.06	6.2	0.2	‡
ALR 26G	Clinopyroxene		3.93×10^{-8}	0.09	6.2	0.3	‡
Jan Mayen							
JM 151A	Olivine	Ankaramite	—	—	6.3	0.5	§
	Clinopyroxene		9.7×10^{-9}	0.4	6.8	0.3	
Prince Edward							
WJ 21E	Olivine	Ankaramite, top of western escarpment	2.33×10^{-8}	0.05	7.4	0.2	
Kilauea							
H66050	Olivine	Picrite, crater wall	5.8×10^{-9}	0.3	14.0	1.4	¶
Puna 2	Glass	Tholeiite, East Rift	1.51×10^{-7}	0.03	14.7	0.5	#
Puna 8	Glass	Tholeiite, East Rift	1.88×10^{-7}	0.04	14.5	0.3	#
Loihi Seamount							
KK 23-3	Glass	Tholeiite	6.1×10^{-8}	0.04	23.1	0.8	††
KK 20-4	Glass	Alkali basalt	5.20×10^{-7}	0.14	24.1	0.7	††
KK 18-8	Glass	Tholeiite	2.71×10^{-7}	0.06	31.9	0.7	††

All samples were crushed *in vacuo* except those denoted*, which had the helium extracted by melting *in vacuo* after crushing. All $^3\text{He}/^4\text{He}$ ratios are reported relative to atmospheric (R/R_a) using an atmospheric value of 1.384×10^{-6} . Sample sources: †Dr S. Humphris; ‡Dr A. le Roex; §Dr S. Maaloe; ||Dr W. J. Voerwoerd, see ref. 50; ¶Dr S. O. Agrell; #Dr J. Moore, see refs 51 and 23, sample numbers refer to Table 2 of ref. 51; ††Dr D. Clague, see ref. 52.

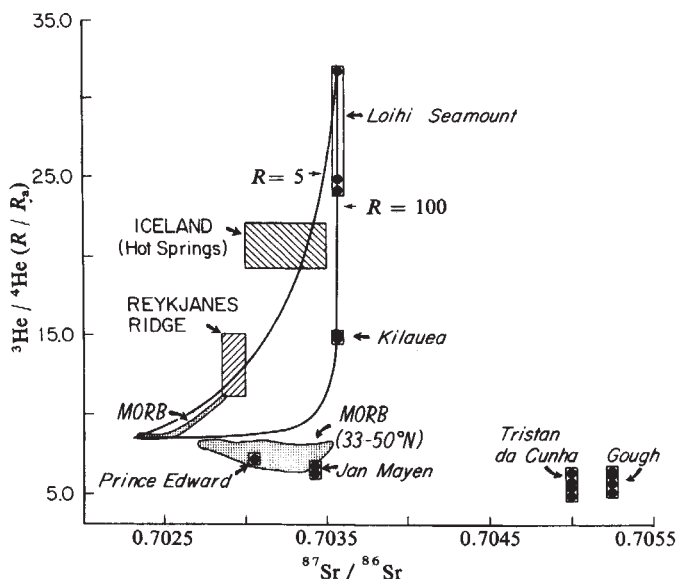


Fig. 1 $^3\text{He}/^4\text{He}$ (relative to atmosphere) plotted against $^{87}\text{Sr}/^{86}\text{Sr}$ for oceanic volcanic rocks. Data sources: Mid-Atlantic Ridge (MORB)¹⁶; Reykjanes Ridge^{26,40}; Iceland^{26,27,40,41}; Kilauea this study and ref. 23; Loihi Seamount, this study; Prince Edward, this study; Jan Mayen this study and ref. 42; Tristan da Cunha this study and ref. 3; Gough Island, this study. Note that the Gough, Prince Edward, MORB, Kilauea, and Loihi samples had helium and strontium isotopic analyses run on the same samples; in all other cases, the fields indicated represent ranges of values for similar samples from the references listed above. For the Icelandic hot springs, the highest reported $^3\text{He}/^4\text{He}$ ratios^{26,27} were selected to minimize atmospheric effects. The highest terrestrial $^3\text{He}/^4\text{He}$ ratios ($37\times$ atmospheric) reported by Kaneoka and Takaoka¹⁵ are not plotted because they do not report $^{87}\text{Sr}/^{86}\text{Sr}$ ratios. The two mixing lines were calculated assuming component 1 has $^3\text{He}/^4\text{He} = 8.5\times R_a$, $^{87}\text{Sr}/^{86}\text{Sr} = 0.70230$ and component 2 has $^3\text{He}/^4\text{He} = 32.0\times R_a$, $^{87}\text{Sr}/^{86}\text{Sr} = 0.70358$. As shown by Langmuir *et al.*²⁸, curvature is determined by the ratio R , where in the present case:

$$R = \frac{^4\text{He}_1 \cdot ^{86}\text{Sr}_2}{^4\text{He}_2 \cdot ^{86}\text{Sr}_1}$$

with age of the Earth. This contrasts with continental crust, which is greatly enriched in U and Th and degassed of ^3He values for the North Atlantic, which are discussed in detail elsewhere¹⁶, and literature values for Icelandic hot springs and the Reykjanes Ridge^{25,26}.

To explain these trends, we can immediately eliminate *in situ* (post-eruptive) decay of U and Th isotopes to produce the low $^3\text{He}/^4\text{He}$ trend, as all the samples are of essentially zero age and the phenocrysts analysed contain low U contents. This is also supported by the analyses of olivine and clinopyroxene from the same sample, which yielded similar results. We believe that the trends in Fig. 1 are most easily accounted for by three component mixing. One component, with $^3\text{He}/^4\text{He}$ of $\sim 1.17\times 10^{-5}$ ($8.5\times$ atmospheric) and $^{87}\text{Sr}/^{86}\text{Sr}$ of ~ 0.7025 , would then be identified as the 'typical MORB' reservoir. A second component, characterized by high $^3\text{He}/^4\text{He}$ and higher $^{87}\text{Sr}/^{86}\text{Sr}$ ratios, would be consistent with a more primitive source derived from 'mantle plumes', as has been suggested for Hawaii and Iceland^{15,27}. The third component is characterized by low $^3\text{He}/^4\text{He}$ and high $^{87}\text{Sr}/^{86}\text{Sr}$ ratios, as indicated by the results for Tristan and Gough.

The curve defined by the MORB-Loihi trend in Fig. 1 is consistent with mixing, as ratio-ratio plots do not always display straight lines for binary mixing²⁸. Several mixing lines (calculated for different He and Sr concentrations in the end-members) are shown for reference in Fig. 1. The helium isotopic ratio of a possible primordial end-member (either 'planetary' or solar) is shown in Fig. 2. Because of the high He/(Th + U) ratio characteristic of the Sun and meteorites, there is little change in the $^3\text{He}/^4\text{He}$ ratio (due to addition of radiogenic ^4He)

during formation, resulting in quite low present-day $^3\text{He}/^4\text{He}$ ratios (see Fig. 2). The primordial end-member would then lie between the Loihi seamount point and bulk earth as plotted in Fig. 2. Because it is not clear that there is a genetic relationship between the Earth and the meteorites, or whether any undifferentiated terrestrial mantle still exists, the true end-member must remain uncharacterized¹⁶.

The low $^3\text{He}/^4\text{He}$ samples for Gough and Tristan da Cunha require mixing with a reservoir that has been enriched in Th and U relative to ^3He for time periods long enough to lower the $^3\text{He}/^4\text{He}$ ratio. The time required depends on the $^3\text{He}/(\text{Th} + \text{U})$ ratio¹⁶. Our data cannot distinguish between seawater, subducted oceanic crust plus sediment, or old continental crust as a source for this component. As shown in Fig. 2, any of these would serve as an appropriate end-member if the mixing hypothesis is used to explain the variations. However, given the Sr-Nd correlation and the expected effect of seawater addition on these isotopes, seawater seems unlikely²⁹. Seawater also contains small quantities of helium relative to basaltic melts, so addition of large quantities would be required to lower the $^3\text{He}/^4\text{He}$ ratio, and would result in extreme variations in $^{87}\text{Sr}/^{86}\text{Sr}$.

The suggestion³⁰ that an important source of isotopic variation in oceanic basalts is contamination from the oceanic crust through which the eruptive basalts must pass is plausible in that oceanic crust should separate ^3He from Th and U by degassing. However, this mechanism seems unlikely for several reasons. First, volcanics on Tristan da Cunha, Gough, Jan Mayen and Prince Edward are erupted through oceanic crust that is much younger (and thinner) than the crust beneath Hawaii, and yet the $^3\text{He}/^4\text{He}$ ratios are lowest. If contamination were significant, one would expect the oldest, most radiogenic crust to lower the ratio the most. Second, special conditions would be required to lower the magmatic $^3\text{He}/^4\text{He}$ ratio by this mechanism. The contaminating crust must have lost most of its initial He, and must have produced (and retained) substantial quantities of radiogenic ^4He . The generally low U and Th contents of unaltered oceanic crust require extreme enrichment in these

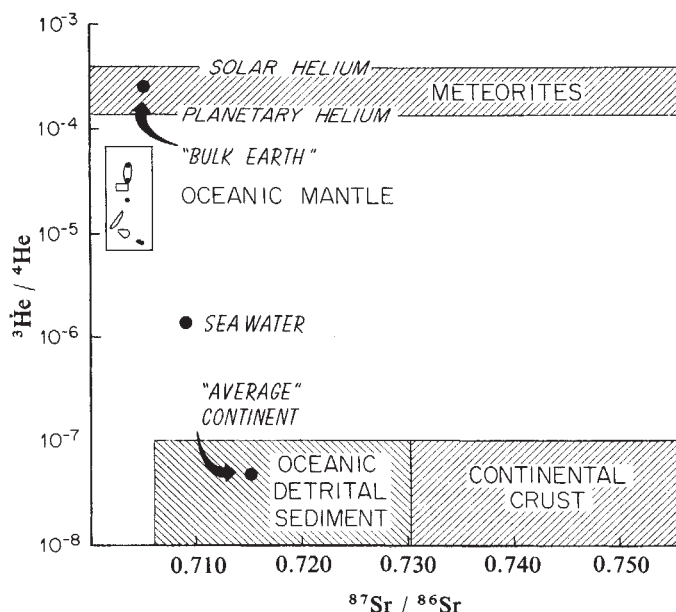


Fig. 2 Simplified plot of $^3\text{He}/^4\text{He}$ versus $^{87}\text{Sr}/^{86}\text{Sr}$ for terrestrial materials and meteorites. Data sources: chondrites^{43,44}; bulk earth^{3,44}; oceanic mantle (see Fig. 1); seawater^{45,46}; continental shield^{10,47}; oceanic detrital sediments⁴⁸; and average crust^{6,49}. Oceanic detrital sediments, and average crust are assumed to have the same range of $^3\text{He}/^4\text{He}$ ratios as continental gases¹⁰. We have also ignored meteoritic spallation helium, which can have $^3\text{He}/^4\text{He}$ ratios higher than 10^{-4} . The upper boundary for the oceanic mantle field is defined by helium analyses by Kaneoka and Takaoka¹⁵.

elements to generate enough ^4He in reasonable time periods. This is particularly true for Tristan da Cunha and Gough, which are situated on oceanic crust that is 10–20 Myr old.

One mechanism that cannot be ruled out is the separation of He from U and Th by multiple melting events. For example, if the mantle beneath Tristan da Cunha and Gough were 'enriched' by the addition of a small amount of melt or fluid (equivalent to metasomatism³¹), it is conceivable that helium would be lost by degassing, while the U and Th would be retained. The $^3\text{He}/^4\text{He}$ ratio would then decrease, due to extremely low $^3\text{He}/\text{U}$ and $^3\text{He}/\text{Th}$ ratios; on melting, this enriched mantle would yield low $^3\text{He}/^4\text{He}$ ratios. Trace element analyses from Tristan da Cunha and Gough suggest that the lavas are enriched in Th and U relative to chondrites³², making this feasible. However, the small degree of partial melting, which may generate oceanic island alkali basalts, makes it difficult to determine the source mantle characteristics from the trace element concentrations and ratios³³. In addition, the physical process by which helium is lost from the mantle without melt removal is unclear.

The subduction of altered oceanic crust and sediments into the mantle has been suggested as an explanation for some of the Sr–Nd–Pb variations^{7,8,34}, and is quite consistent with our results for Tristan da Cunha and Gough. If the low $^3\text{He}/^4\text{He}$, high $^{87}\text{Sr}/^{86}\text{Sr}$ mantle source region is produced by adding subducted crust into the mantle and then remelting, it is important to evaluate the effect on helium isotopes. The $^3\text{He}/^4\text{He}$ ratio that would result from remelting of this material is a function of the initial helium content of the crust, the amount of degassing it has undergone (both before and during subduction), the helium content of the mantle to which it is added, and the relative proportions of the two mantle types. These formidable uncertainties make a quantitative treatment impossible; however, there is clearly sufficient U and Th present to produce the observed variations, depending on the physical processes occurring. For example, in 200 Myr, $4 \times 10^{-6} \text{ cm}^3 \text{ g}^{-1}$ of radiogenic ^4He will accumulate in oceanic crust having an average U content of 100 p.p.b. (parts per 10^9) and $\text{Th}/\text{U} = 2$ (ref. 35). If we take $1 \times 10^{-5} \text{ cm}^3 \text{ g}^{-1}$ as an upper limit of the initial helium content of oceanic crust, the $^3\text{He}/^4\text{He}$ ratio in this crust will decrease by at least 30% in 200 Myr. In addition, it is possible that hydrothermal alteration adds U to the crust, making 100 p.p.b. a lower limit^{7,36,37}. The other extreme is an oceanic crust that degasses completely on formation, leaving only radiogenic ^4He to remix with the mantle on resubduction. Craig and co-workers³⁸ have interpreted low $^3\text{He}/^4\text{He}$ ratios in present-day back-arc volcanic systems to be a result of mixing between the helium in the downgoing slab and the helium in the underlying mantle, which suggests that degassing continues after crustal formation. In both cases, the $^3\text{He}/^4\text{He}$ will decrease to some extent, depending on the mixing ratio of the two components. Clearly, there is sufficient U and Th to lower the $^3\text{He}/^4\text{He}$ ratio significantly even before subduction. If the subducted crust remains in the mantle for long periods, as suggested by Hofmann and White⁷, substantial quantities of radiogenic ^4He may accumulate.

All three mechanisms described above (crustal contamination, multiple melting, and remelting of subducted crust in the mantle) could conceivably produce the low $^3\text{He}/^4\text{He}$ trend defined by the Tristan da Cunha and Gough points. At present, we believe that the subducted crust hypothesis most easily accounts for the trend. In contrast to the other two processes, subduction is a commonly observed phenomenon. The mechanism for separation of He from U and Th is degassing of the oceanic crust, another presently observable phenomenon¹⁴. Further, the allowed time periods and U enrichments necessary to lower the $^3\text{He}/^4\text{He}$ ratio are geologically quite reasonable.

If all the high $^3\text{He}/^4\text{He}$ islands are produced by two component mixing with the same end-members, then various $^4\text{He}/^{86}\text{Sr}$ ratios are required, as this will determine the curvature of the mixing line. For example, if the Kilauea isotopic signature is derived by mixing the two end-members shown in Fig. 1,

then the high $^3\text{He}/^4\text{He}$ end-member must have higher Sr contents or lower He contents (or both, see Fig. 1). Alternatively, another end-member may be involved. Note that Tatsumoto³⁵ observed different Pb isotopic compositions for each of the five sub-aerial Hawaiian volcanoes. He suggested that the linear trend, defined by the Hawaiian volcanoes, on the $^{207}\text{Pb}/^{204}\text{Pb}$ against $^{206}\text{Pb}/^{204}\text{Pb}$ plot was a mixing line. To test the mixing hypothesis, a more detailed study of the Loihi Seamount and the island of Hawaii is underway in our laboratories.

It would appear that $^3\text{He}/^4\text{He}$ measurements, coupled with the other isotopic measurements, are an important discriminant between primordial and 'recycled' mantle source regions. The reason for the large variations is that separation of He and Th + U occurs by degassing, which is not the case for the Rb–Sr, Nd–Sm, and U–Pb systems. Note that the samples lying close to bulk earth on the Nd–Sr correlation line, such as Tristan da Cunha and Gough, are not necessarily representative of undepleted mantle. It is possible to derive the trend by mixing between some crustal components^{9,39} and the depleted MORB reservoir, resulting in a coincidental bulk earth value.

Therefore, on the basis of the helium isotopic information, three distinct mantle reservoirs are required: depleted (the MORB source), undepleted, and recycled. The islands displaying the highest $^3\text{He}/^4\text{He}$ ratios have tholeiitic affinities (Hawaii and Iceland), while the islands consisting of alkali basalts have characteristically lower $^3\text{He}/^4\text{He}$ ratios (Tristan da Cunha and Gough). More detailed sampling is required to confirm this trend.

We thank the following for providing samples: S. O. Agrell, D. Clague, S. Humphris, A. le Roex, S. Maaloe, J. Moore, R. K. O'Nions and W. Voerwoerd; also A. le Roex, G. Thompson, J.-G. Schilling and H. Dick for interesting discussions, and B. Haskell for editorial comments. M.K. thanks the Education Office of the WHOI/MIT Joint Program in Oceanography for their continuing support. This work was supported by NSF grant OCE-7909379 and USGS contract 14-08-001-G541. This is WHOI contribution no. 4955.

Received 24 August 1981; accepted 5 March 1982.

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Scaling rules in rock fracture and possible implications for earthquake prediction

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A major preoccupation in physical sciences has been to interpret macroscopic events from microscopic phenomena. In some cases the change of scale is efficient and fairly easy to perform, but in others it turns out to be difficult and uninteresting. Success or failure is due more to the nature of the events than to the efficiency of the theoretical methods used. Some macroscopic phenomena have their origin in a microscopic organization which can be transferred to larger scales whereas others attain their structure on the macroscopic scale itself. Thus before applying scaling laws techniques^{1–4} one must ensure that embedded scales are suggested by physical observations. That this seems to be the case for the fracture of rocks is supported by geological, seismological and rock mechanics observations. We have therefore built a very simple model based on scaling laws which yields a criterion for fragility at different scales and views rupture as a critical point. We use this model here to outline a general approach to earthquake prediction.

Fracturing occurs in rocks at all scales, from the microscale (microcracks) to the continental scale (megafaults), and the geologist can equally well observe embrittlement and rupture phenomena under the microscope as on satellite photographs (Fig. 1). But are the various scales of fracture related to one another? The following observations suggest they are: (1) field geologists know that the great faults of the crust—such as the San Andreas fault—actually consist of anastomosed faults, sometimes arranged *en échelon*, thus weakening a whole domain of the crust, down to variable depths⁵. (2) Seismologists who study source phenomena often have to introduce in their models

complex rather than single faults, each one contributing to the observed radiation pattern^{6,7}. (3) Rock mechanicians, when studying fracture in the laboratory, observe that it is preceded by the concentration of a swarm of microfissures which are themselves the result of an accumulation of microcracks^{8–11}. From this set of observations one can suggest that fracture at the macroscopic scale is a consequence of accumulations of ruptures at lesser scales.

This hypothesis has been actually adopted by Brace and his students^{8,9,11,12}. They have submitted rock samples to progressive triaxial loading ($\sigma_1, \sigma_2, \sigma_3$) and studied the increase of microcrack density with increasing load. This increase seems to be the result of two distinct processes: (1) the nucleation of new cracks, that is, the birth of new rupture points in the material; and (2) the growth of pre-existing cracks. In fact, as noted by Brace *et al.*⁸ and Tapponnier and Brace¹¹, the nucleation seems to occur most often from a pre-existing crack. The distinction between the two processes is thus subtle and the increase in crack density can be considered to be ruled by a single phenomenon with a given activation energy.

The law governing the increase of microcrack density with deviatoric stress ($\sigma_1 - \sigma_3$) depends strongly on the confining pressure. But what seems to occur generally is that the macroscopic fracture is not preceded by an accelerated growth of microcrack density as measured over the whole sample. When the sample is examined at different scales one observes that cracks collect in some regions, but that these microscopic regions are roughly homogeneously distributed in the medium, even when the rupture threshold has been reached. On the other hand, the larger the scale the stronger is the spatial heterogeneity. The heterogeneity reaches, of course, a limit which determines the fracture itself whose orientation follows the laws established by Anderson¹³.

We will now try to explain those observations with a simple renormalization group (RG) model and examine possible implications of the model for earthquake prediction.

For each elementary domain of rock (say 100 μm), we define two states: when the local microcrack density in the domain is greater than some critical value, it is considered as fragile (f); otherwise it is considered as sound (s).

As shown elsewhere (for example, see ref. 11), the mean microcrack density d depends linearly on ($\sigma_1 - \sigma_3$) (for a given σ_2), but the local density varies considerably within the sample. The probability for an elementary domain to be fragile, p , is directly proportional to d and thus linearly related to ($\sigma_1 - \sigma_3$) (for a given σ_2):

$$d = d_0 + b(\sigma_1 - \sigma_3)$$

$$p = ad = ad_0 + ab(\sigma_1 - \sigma_3) \quad (1)$$

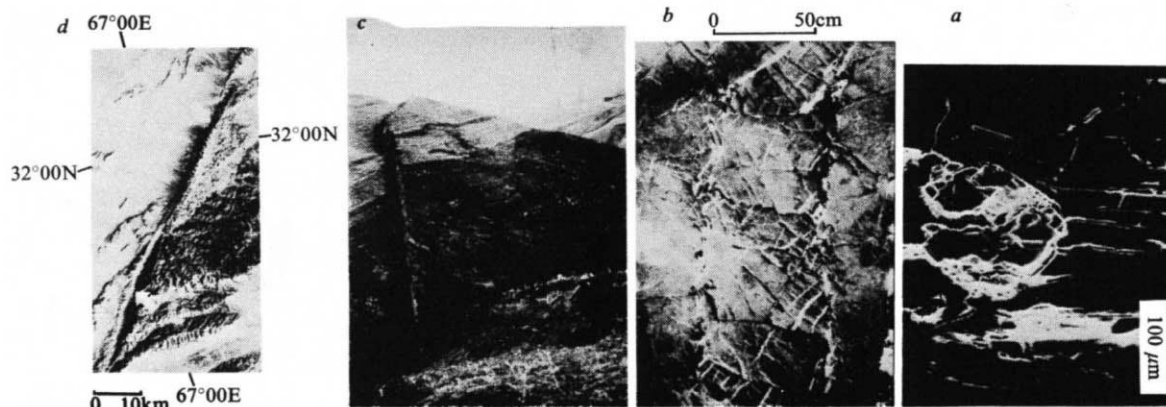


Fig. 1 Examples of fracture at different scales. *a*, Microcracks in quartz grains are induced by intense cracking in magnetite and plastic flow in biotite. (Westerly granite, fracture stress, 35 bar of confining pressure, room temperature.) *b*, Tension gashes, stylolites and micro shear faults in horizontal Mesozoic limestones (near Les Matelles, in Languedoc, France). The microstructures combine to form a fault zone at a larger scale. *c*, Master fault of the El Asnam, Algeria, earthquake (magnitude = 7.3, 10 October 1980). In the hills north of El Attaf, the break, several kilometres long, has up to 4 m of vertical throw. *d*, Landsat image of the Chaman strike-slip fault south-west of the Katawaz basin near the border between Afghanistan and Pakistan. The fault system, ~1,200 km long, may have accommodated as much as 500 km of left lateral displacement of India past Afghanistan in the past 40 Myr.

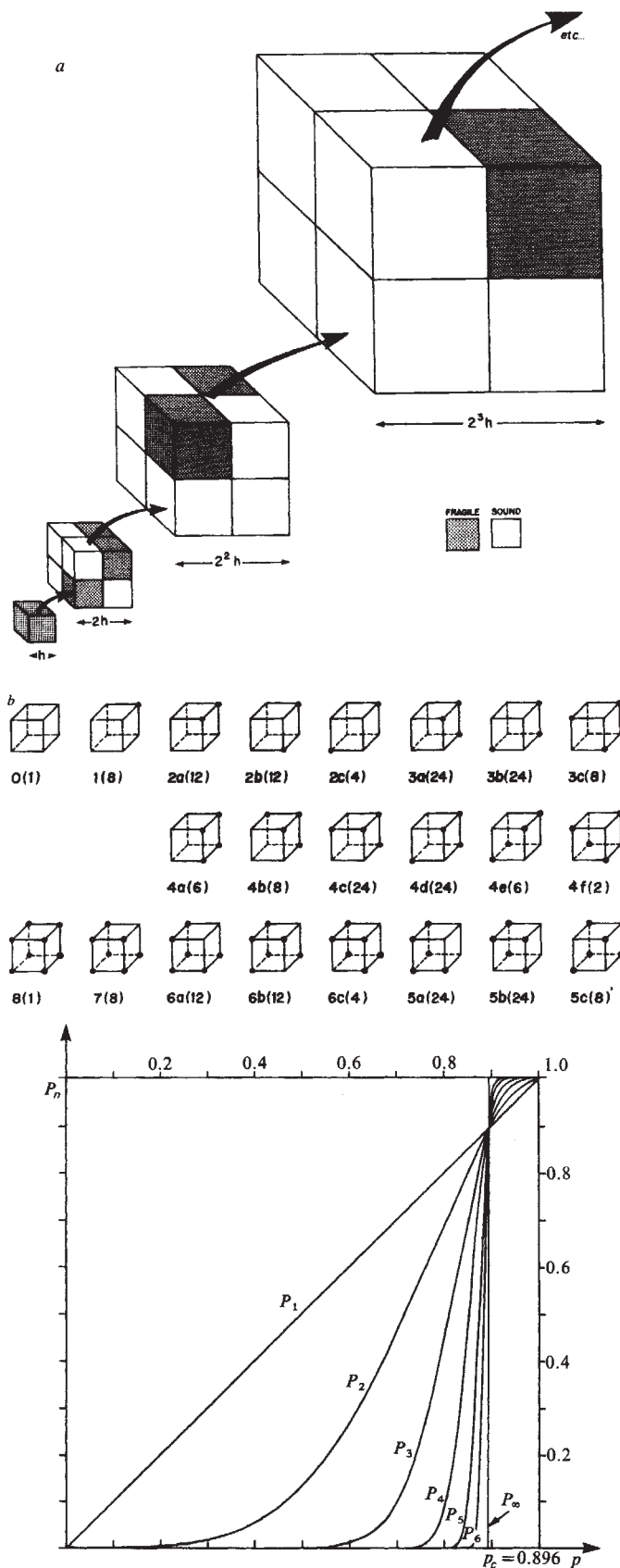


Fig. 2 *a*, Illustration of the renormalization group model. *b*, Now an n -order cube is depicted as a simple cube with its corners representing the eight constitutive $(n-1)$ -order cubes. Each corner is marked by a solid dot when the corresponding $(n-1)$ -order cube is fragile. This defines 22 configurations labelled 0, 1, 2a, ... 8 with their multiplicities in parentheses. The probability for a configuration with m corners fragile and multiplicity μ is: $\mu(P_{n-1})^m(1-P_{n-1})^{8-m}$. *c*, Probability P_n for an n cube being fragile plotted against the microscopic probability p .

Parameters d_0 and b depend on σ_2 , whereas a can be considered purely geometrical.

Let us now develop a criterion for fragility at different scales. Let the elementary domain of order 1 be a cube of unit side (for example, 100 μm). Let the order of 2 cube be composed of 2^3 elementary order of 1 cubes, and so on (Fig. 2a).

In terms of $(n-1)$ cubes (we write n cube for the order of n cube), there are $2^8 = 256$ different n cubes. Taking the symmetry group of the cube into consideration, this number reduces to 22 topologically different configurations, each with a given multiplicity (Fig. 2b). An n cube will be considered sound whenever there is a 'pillar' of sound $(n-1)$ cubes that links two opposite faces. We thus consider as fragile the configurations labelled 4f, 5c, 6b, 6c, 7 and 8 in Fig. 2b. If P_n is the probability for an n cube being fragile, then:

$$P_1 = p, \quad P_2 = \Pi(p), \quad \dots, \quad P_n = \Pi(P_{n-1}) \quad (2)$$

with

$$\Pi(x) = 2x^4(1-x)^4 + 8x^5(1-x)^3 + 16x^6(1-x)^2 + 8x^7(1-x) + x^8$$

The polynomial $\Pi(x)$ has the following simple properties: $\Pi(x) = x$ for $x = 0$, $x = p_c = 0.896$ and $x = 1$. It monotonously increases with x on $[0, 1]$, being less than x on $]0, p_c[$ and greater than x on $]p_c, 1[$. Furthermore, $\Pi'(1) = \Pi'(0) = \Pi''(0) = \Pi'''(0) = 0$. This explains the characteristics of the probability law P_n , which are illustrated in Fig. 2c. A striking feature is how rapidly the curves converge to the (Heaviside) step-function $H(p - p_c)$ with increasing n . For $n = 5$, P_n positively jumps from 0 to 1 when p passes p_c .

This crude model allows one to account very simply for the observations of Tapponnier and Brace¹¹. When counting microcrack density at the lowest scale an increase of this density increases with $(\sigma_1 - \sigma_3)$ is observed, but nothing clearly announces the macroscopic rupture which appears as an 'exogen' discontinuity in a continuous process. The situation is quite different when looking at larger scales: little occurs before p gets to values close to p_c ; then the fragility probability P_n jumps rapidly to values close to 1 and rupture occurs (see Fig. 3). p_c is thus a critical probability, the threshold for crack percolation.

Of course, the fragility criterion we have chosen is arbitrary and oversimplified. Other configurations of $(n-1)$ cubes may be supposed to make the n cube fragile. More refined criteria may be built by considering higher-order scaling steps ($3^3 = 27$, $4^3 = 64$, and so on, elementary cubes instead of $2^3 = 8$). In any case we get a recursion relationship between P_{n-1} and P_n of the form $P_n = \Pi(P_{n-1})$, Π being a polynomial with properties analogous to the ones above. Figure 4 illustrates a case in which the renormalization polynomial $\Pi(x)$ has no fixed point except the trivial ones $x = 0$ and $x = 1$. Although such a choice might seem inappropriate ($P_n \rightarrow 0$ when $n \rightarrow \infty$ for all $p < 1$), it leads to the same conclusions (a step from very low to very high probabilities at the highest scales) due to the fact that there is

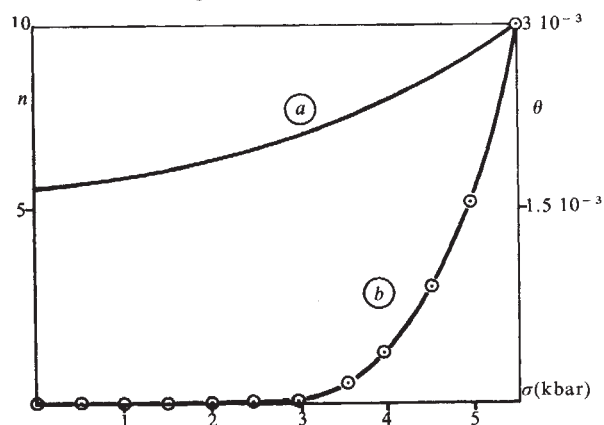


Fig. 3 Schematic representation of some experimental results (adapted from ref. 11). Sample of Westerly granite, confining pressure 500 bar. *a*, Variation with σ of a microscopic property (number of crack intersections/mm, n); *b*, variation with σ of a macroscopic property (volumetric crack dilatancy, θ).

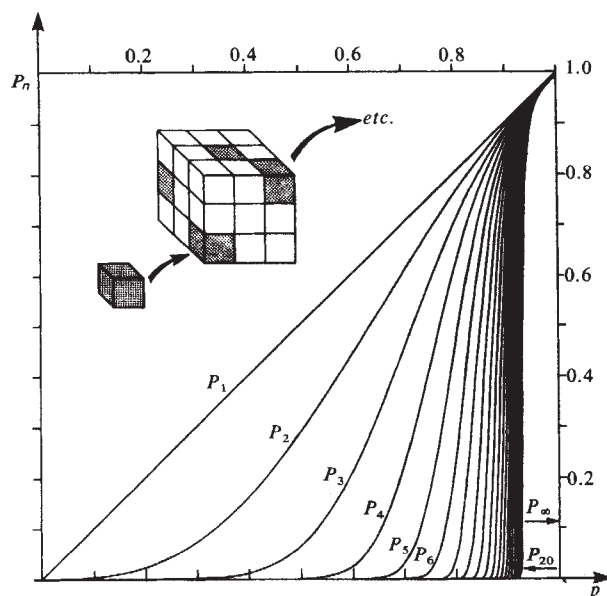


Fig. 4 Now an n cube, made of $27(n-1)$ cubes, is considered as fragile when three fragile $(n-1)$ cubes are aligned along one of the three medians of the n cube. Then $P_1 = p$, $P_2 = \Pi(p)$, $\dots$, $P_n = \Pi(P_{n-1})$ with $\Pi(x) = 3x^3 - 3x^5 + x^7$. With increasing n , the P_n curves sharpen rapidly to a quasi-step, then shift only slowly to the right. The last curve drawn is that for $P_{20}(3^{19} > 10^9)$. This shows that the prediction for megafaulting is not significantly altered by the mathematical absence of a critical probability p_c .

not an infinity of scaling steps from microcracks to megafaults (100 km/100 $\mu\text{m} = 10^9$). On the same basis it is also possible to build 'anisotropic models' in which the fragility is relative to a given direction and varies with this direction. But rather than generalizing, we will now draw some inferences about earthquake prediction from the simple model considered above.

The dilatancy theory for earthquake prediction which has been developed from laboratory experiments^{8,14,15} has had some success but has failed to provide a general solution. Using a similar approach, we now show how to draw, from laboratory experiments, other useful inferences on earthquake prediction.

Earthquakes are fracture phenomena which most often occur on pre-existing sets of faults. Together with many theoretical seismologists we may assume that the reactivation of a fault system is a fracture phenomenon polarized by the existence of the fault itself^{16,17}. Then the approach we propose, which determines a critical fragility, can also apply to earthquakes. One only needs to consider that the macroscopic domain with which our computations are concerned is the domain of the faults.

Earthquakes are probably generated by a strain accumulation under a constant deviatoric stress $\sigma = \sigma_1 - \sigma_3$ rather than by a continuous increase of σ with time. Mogi¹⁸ has proposed that, in a medium submitted to a constant σ , the increase in the probability of occurrence of fracture during the time interval $(t, t+dt)$ does not depend on t and is given by $dp = \mu_0 \exp(\beta\sigma) dt$, where μ_0 and β are constants. We can also consider the more general case when stress is increasing with time:

$$p(t) = p_0 + \int_0^t \mu_0 \exp(\beta\sigma(t)) dt \quad (3)$$

(We may assume, in such conditions, that the microcrack density obeys a zero-order kinetic equation $d'(t) = \delta_0 \exp(\beta\sigma)$, $d(t)$ and $p(t)$ being related by $p(t) = a d(t)$, as above.)

It is then possible to compute the curves $P_n(t) = P_n[\sigma(t)]$ by the recursion formula (2). Of course, the shape of those curves is the same as that in Fig. 4, the parameter along the horizontal axis now being the time. For example, in the experiments of Kranz on samples of Barre granite¹⁹, fracture occurs about 150 s after a stress of 2 kbar has been applied. To get the time parametrization, one has only to replace the $(0, p_c)p$ interval by a $(0, 150 \text{ s}) t$ interval. If σ is supposed to vary with time, a

variable contraction or dilation of the horizontal axis can modify the figure of the $P_n(t)$ curves.

Suppose now we have two physical parameters, (x) and (y) , which depend in a known way on crack density at different scales n_1 and n_2 . It is then possible to predict the critical point $p(t) = p_c$ from the predicted intersection of the two curves $x(t)$ and $y(t)$. So it may be thought that the prediction of earthquakes demands the observation of phenomena characterized by different scales. For example, if one studies seismic waves for periods of 0.1, 1, 10, 100, $\dots$ s, one may think that the values of the corresponding attenuation ratios will tend towards some limit at the critical point (the partial failure of the (V_p/V_s) ratio prediction technique may come from the fact that in some circumstances this ratio depends on the scale but not in others, whether pores containing water in the rock are connected or not).

The crude model we propose here may be tested in two ways. First by studying in the laboratory the variation of crack density at different scales as $(\sigma_1 - \sigma_3)$ increases; second, by studying the variations of some physical properties which depend on crack density at different scales, especially in the vicinity of fracture.

Despite the extreme simplicity and the limits of our approach, if our basic conjecture is correct, a set of prediction methods could be built up.

We thank T. Madden, P. Tapponnier and V. Courtillot for fruitful discussion. P. Tapponnier provided the photographs in Fig. 1.

This is Institute de Physique du Globe contribution no. 578.

Received 6 January; accepted 11 March 1982.

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Cobalt in north-east Pacific waters

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Significant understanding has been gained recently about the biogeochemical cycling of trace metals in the ocean. This knowledge has mostly resulted from the accurate measurement of dissolved species in oceanic water columns. We report here that cobalt's vertical distribution is similar to that exhibited¹⁻³ by Mn; that is, its surface enrichment/deep depletion (Fig. 1). However, amounts of Co (1–7 ng l⁻¹) are ~10–20 times less than those for Mn (Table 1), as might be expected from crustal abundance estimates⁴ for these elements (Mn=950; Co=25 μg per g). The similarity between Mn and Co profiles implies the same biogeochemical pathways. The Co excess in nearshore surface waters probably results from continental weathering input processes, as suggested by the remarkable Co-salinity mirror-image relationship shown in Fig. 1, and the Co-salinity scatter diagram in Fig. 2a. The steady decrease in Co concentrations also indicates that Co is usually scavenged rather than regenerated at depth, as is the case with Mn (Fig. 1; Table 1).

Table 1 Dissolved Co levels observed off central California (36° 30' N 123°05' W) in June 1981

Depth (m)	OE Co 1 (ng l ⁻¹)	OE Co 2 (ng l ⁻¹)	$\bar{X}$ OE Co (ng l ⁻¹)	CX Co* (ng l ⁻¹)	CX Mn† (ng l ⁻¹)
0.2 raft	7.0	6.9	7.0	—	—
10	6.6	6.2	6.4	1.8	130
30	6.8	7.3	7.0	—	160
50	6.6	6.0	6.3	5.1	—
100	5.8	5.9	5.8	4.7	62
200	4.5	3.6	4.0	4.0	40
300	3.9	3.9	3.9	3.3	42
400	3.6	3.4	3.5	2.2	49
520	25.0‡	2.9	2.9	1.1	58
700	2.2	1.8	2.0	2.0	50
980	2.1	1.6	1.8	2.0	45
1,160	1.5	2.1	1.8	2.0	43
1,900	1.8	1.5	1.6	1.9	42
2,350	1.4	1.4	1.4	1.6	31

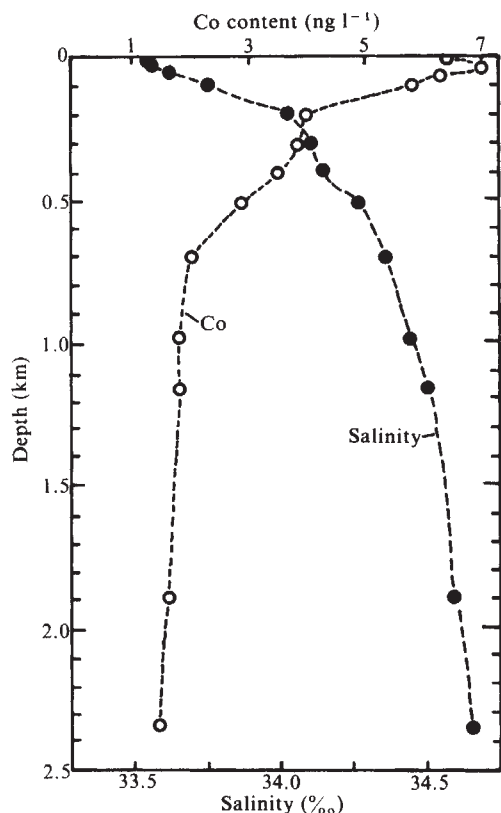
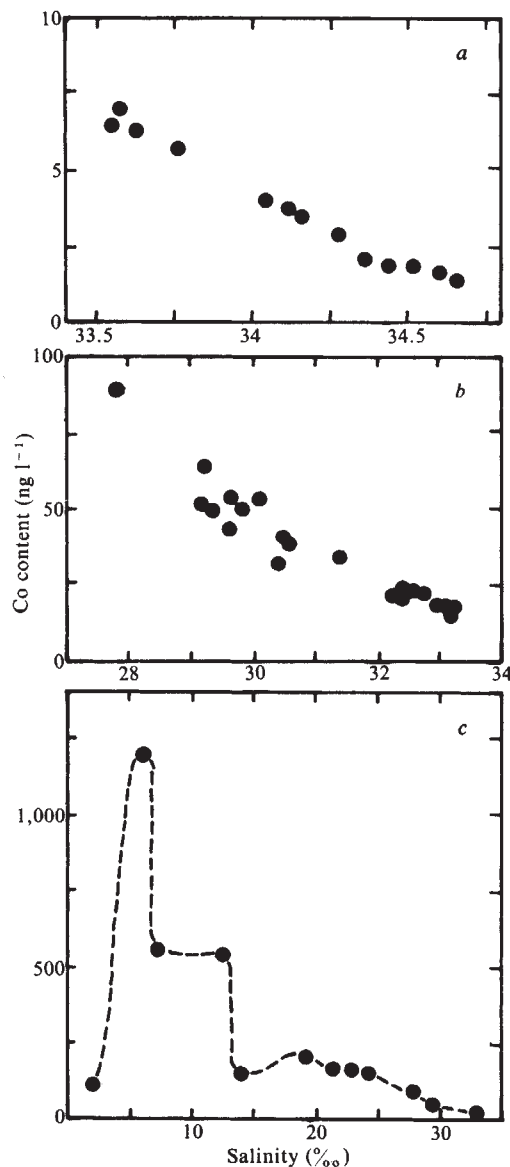
Cobalt was preconcentrated in two replicates (OE Co 1, OE Co 2) using organic extraction. Replicate values were averaged ($\bar{X}$ OE Co) and are compared with Co amounts obtained using chelex preconcentration (CX Co). Manganese data (CX Mn) for these samples are also shown. Water was filtered using 0.45 μ m pore size nucleopores.

* Chelex at pH 6.

† Chelex at pH 8.

‡ Contaminated.

In comparison with those in oceanic waters, Co values can be very high in estuaries. We measured dissolved Co amounts varying between 50 and 1,200 ng l⁻¹ in north San Francisco Bay on a Sacramento River/Golden Gate transect during a period of high freshwater flow in March of 1980. The highest concentrations were observed at three stations with salinities between 6.2 and 12.4‰; that is 1,200, 550 and 530 ng l⁻¹ (Fig. 2c). Cobalt levels immediately outside the above salinity range were markedly lower: 120 and 140 ng l⁻¹, at salinities of 2.2 and 13.8‰, respectively. Amounts of Co more or less steadily decreased in the remainder of the salinity range sampled; that

**Fig. 1** Salinity (●) and mean organic extraction Co values (○) versus depth (see also Fig. 2a).**Fig. 2** Cobalt-salinity relationships observed a, off central California at depths of 0.2–2,350 m; b, in near-surface waters immediately outside San Francisco Bay; c, in San Francisco Bay. Correlation coefficients for data in a and b are -0.93 and -0.99, respectively.

is, from 200 ng l⁻¹ at 19.3‰ to 40 ng l⁻¹ at 33‰ (Fig. 2c). A similar Co-salinity relationship was observed for near-surface waters collected outside San Francisco Bay (Fig. 2b). The slight curvature in the plotted points suggests the possibility of Co removal processes occurring within this salinity range, as well as conservative mixing.

The reasons for the very high dissolved Co levels in the San Francisco Bay intense mixing zone (6–12‰ salinity range) waters are not understood. Although the data suggest Co adsorption-desorption reactions occurring with suspended particulates, the exact nature of these reactions is unknown. River-estuarine chemistry is very complicated, involving many competing processes, and is beyond the scope of this paper. Nevertheless, scientists dealing with this complex subject also report similarities between Co/Mn geochemistry⁵.

We compared our Co distribution findings with those reported previously^{6–13}, and in general, values for estuaries and rivers are similar to those we report for near-shore and San Francisco Bay waters, while quantities for various oceanic waters are usually considerably higher. Because most of these data were reported well before the adoption of clean techniques, it is logical to suspect contamination problems for many of the

previously reported oceanic samples, especially those collected at depth.

We avoided contamination by using the same collection, filtration and analytical procedures that we had previously used (with Bruland) for other elements¹⁴. The organic extraction (APDC, DDDC/chloroform) preconcentration method had yields of 97–98% when checked with carrier-free ⁵⁷Co, and the precision for two replicates was generally excellent (Table 1). The use of another preconcentration technique (chelex ion exchange at pH 6) resulted in similar values (within 1.3 ng) with the exception of the 10 and 520 m samples (Table 1). Analyses of eight individual blanks for the organic extraction procedure resulted in a mean and standard deviation of 0.3 ± 0.04 total ng Co in comparison to 0.6 total ng found in the lowest sample. Cobalt was not detected in the chelex blanks.

In conclusion, the oceanic vertical distribution of dissolved Co appears to be very similar to that of Mn, at least as we now know it. Great variability in near-shore surface waters can be expected and, as is the case with Mn, oceanic removal processes are probably rapid, with resulting residence times in tens of years. This can be illustrated by substituting a world ocean Co

concentration of 1 ng l^{-1} in place of the 23 ng l^{-1} used by Bewers and Yeats¹⁵ in their estimates of Co residence times. The results are 52 yr using stream input data and 34 yr using sedimentation rate data.

If the Mn–Co similarity is also true for open-ocean waters, much lower Co levels ($\sim 1.0 \text{ ng l}^{-1}$) can also be expected in oligotrophic surface waters, judging by Mn data ($\sim 35 \text{ ng l}^{-1}$) reported by Landing and Bruland². Because Co is an important element in many essential biological compounds¹⁶, notably the central atom of vitamin B12, the low amounts of this element in the environment suggest the possibility of its being a limiting nutrient in some conditions, or at least that the massive amounts ($5 \mu\text{g l}^{-1}$) routinely added to phytoplankton culture media are unnecessary.

We thank S. Fitzwater, S. Tanner and C. Hunter and the captain and crew of R/V *Cayuse* for their help during our Cobalt I cruise. This research was supported by grants from the NSF Biological Oceanography (OCE 80-03200) and Marine Chemistry (OCE 79-09431) programmes, the Ocean Minerals Corporation, and the US Environmental Protection Agency (CR 8077110020).

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Beryllium in the water column of the central North Pacific

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The existence of the cosmogenic radioisotope of beryllium, ¹⁰Be ($t_{1/2} = 1.5 \text{ Myr}$), was first confirmed^{1,2} almost 25 yr ago. Subsequently, Merrill *et al.*³ worked out the sedimentary geochemistry of the stable isotope of the element, ⁹Be. The geochronological potential of ¹⁰Be could not be exploited, however, because of its extremely low abundance (global average production rate $1.08 \text{ atoms cm}^{-2} \text{ min}^{-1}$)⁴ and the difficulties associated with the analysis of the stable species ⁹Be, particularly in natural waters. This situation is changing dramatically with the application of accelerator techniques to the detection of the radioisotope⁵. It thus becomes important to obtain a detailed understanding of the geochemistry of ⁹Be since the potential use of the stable isotope for normalization purposes would significantly enhance the usefulness of the radioactive species in dating studies. We report here the first detailed profile for ⁹Be in the oceanic water column.

Samples were obtained at 30°N , 158°W over MANOP site R (Manganese Nodule Program Red Clay Study Area) north of Hawaii using clean oceanographic procedures. One litre samples were collected in acid-cleaned linear polyethylene containers and immediately spiked with 4 ml of doubly distilled 6M HCl to bring the pH to ~ 1.8 . Laboratory experiments with standards indicate that there is no loss of beryllium in these storage conditions. The analyses were made by gas chromatography with electron capture detection of the volatile trifluoroacetylacetonate complex of the element. This was formed in the seawater solution at pH 5.4 and extracted into high-purity benzene (details of the method will be reported elsewhere). The detection limit using a 200 ml sample aliquot is about 2 pmol kg^{-1} ($2 \times 10^{-12} \text{ M}$); the precision (1σ) is 5.5% at 20 pmol kg^{-1} .

The profile is presented in Fig. 1 together with the associated hydrographic measurements. The numerical data are listed in

Table 1. The distribution of beryllium in the water column displays the nutrient-like surface depletion and deep enrichment observed for many of the other accurately determined

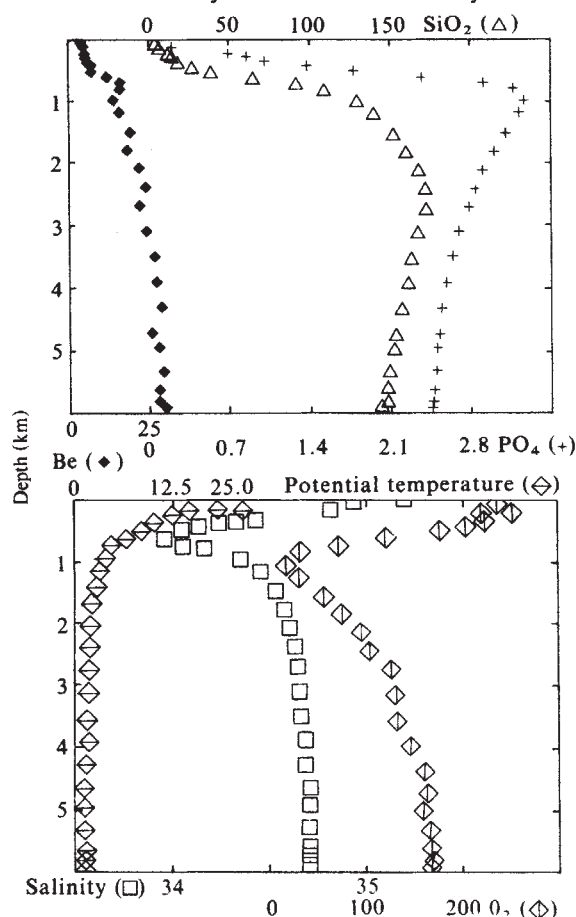


Fig. 1 The beryllium profile from MANOP R in the Central North Pacific (30°N , 158°W). Also shown are the distributions of silicate, phosphate, potential temperature, salinity and oxygen.

Table 1 Beryllium and ancillary data from MANOP R (30° N, 156° W)

Depth (m)	Potential temperature (°C)	%S	O ₂ (μmol kg ⁻¹)	PO ₄ (μmol kg ⁻¹)	Si (μmol kg ⁻¹)	Be (pmol kg ⁻¹)
40	24.64	35.209	244	0.06	2.6	4.0
70	21.60	34.939	253	0.08	3.8	5.0
110	18.72	34.826	241	0.22	5.0	4.0
206	14.67	34.407	218	0.71	10.8	5.0
266	12.68	34.330	219	0.87	13.6	5.4
336	11.30	34.255	221	1.02	17.5	6.0
417	9.96	34.126	206	1.37	26.1	7.0
497	8.04	34.050	178	1.78	38.4	7.0
593	6.15	33.985	122	2.37	64.0	12.0
691	4.75	34.058	74	2.91	90.9	16.0
789	4.30	34.179	32	3.17	108.9	16.0
986	3.67	34.364	20	3.26	129.2	14.0
1,182	3.15	34.462	30	3.22	139.5	16.0
1,505	2.53	34.542	57	3.10	151.3	19.0
1,804	2.17	34.588	76	3.00	159.2	18.0
2,102	1.92	34.618	91	2.90	166.7	22.0
2,401	1.72	34.640	103	2.84	170.5	24.0
2,699	1.61	34.651	126	2.78	171.0	22.0
3,101	1.32	34.665	130	2.69	165.3	24.0
3,508	1.22	34.671	132	2.63	161.7	26.0
3,915	1.16	34.679	147	2.58	159.7	27.0
4,322	1.12	34.684	162	2.55	155.8	28.0
4,730	1.09	34.687	166	2.52	151.7	25.0
4,921	1.08	34.686	162	2.50	150.4	28.0
5,314	1.07	34.688	167	2.49	147.7	29.0
5,608	1.05	34.689	166	2.48	146.7	28.0
5,805	1.05	34.690	168	2.47	146.1	28.0
5,903	1.05	34.689	167	2.46	145.1	30.0

trace elements. The mixed layer value is 4 pmol kg⁻¹; the concentrations rise through the main thermocline to values of 25–30 pmol kg⁻¹ in the deep and bottom waters. Although there may be a secondary maximum associated with the oxygen minimum at 900 m (Fig. 1), the sampling density is inadequate to resolve it fully.

MANOP R is located beneath the tongue of Antarctic Bottom Water that penetrates north around Hawaii⁶. It is the hydrographic feature responsible for the induced maximum in the silica profile that characterizes the North Pacific Deep Water (Fig. 1). The beryllium profile, in contrast to those of the nutrients, increases with depth. The potential temperature–

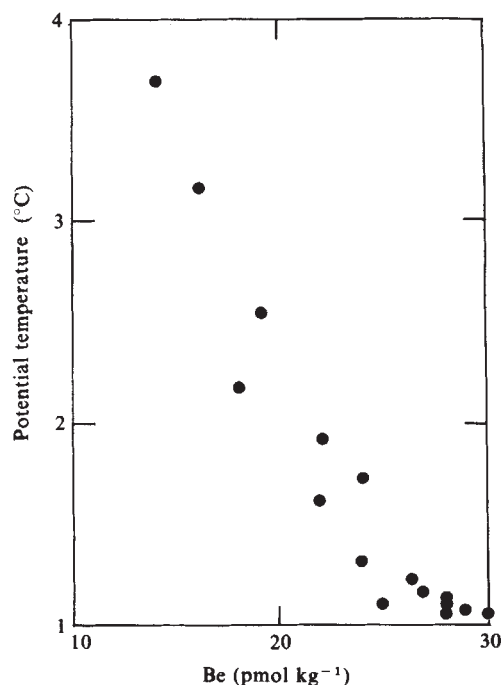


Fig. 2 The beryllium–potential temperature relationship for the deep and bottom waters.

concentration relationship below 1,000 m (Fig. 2) is concave-up indicating mid-water scavenging⁷ as has been observed for copper⁸. The fit to a simple one-dimensional diffusion advection model⁷ gives a value for the ratio of the scavenging rate, J , to the vertical advection velocity, w , of $J/w = 2.49 \times 10^{-3}$ pmol kg⁻¹yr⁻¹m⁻¹ with a standard error of 1.90. Assuming that the upwelling velocity is 3 m yr⁻¹ (ref. 7) then the scavenging rate, J , is 7.5×10^{-3} pmol kg⁻¹yr⁻¹. The half life with respect to scavenging is thus estimated to be 1,850 yr. As the concentration of beryllium increases with depth there must be a source of input from the bottom to maintain the profile against removal by scavenging. This can be crudely estimated⁹ at 1.5 pmol cm⁻²yr⁻¹. Assuming an effective pore water diffusion coefficient of 10^{-6} cm²s⁻¹ then this flux requires a gradient in the sediments of ~ 60 pmol kg⁻¹cm⁻¹. As in the case of copper, such high gradients cannot extend very deep into the sediments because the solubility of any existing beryllium compounds will be exceeded¹⁰. Thus a strong source is required near the surface.

Beryllium exists in the oceans at concentrations as low as those reported for any other trace element (see Fig. 3). The levels in rivers, however, are substantially higher. Merrill *et al.*³ reported values of a few nmol kg⁻¹ (10^{-9} M), which we have confirmed. Three samples from the main channel of the lower Amazon give values of 1.6, 1.7 and 1.8 nmol kg⁻¹, while an estimate from the mouth of the Orinoco is 610 pmol kg⁻¹. The Yangtze gives lower values: 140 pmol kg⁻¹ at the mouth and 150 pmol kg⁻¹ at Wuhan in the central part of the flood plain. If the global average value is taken as 1 nmol kg⁻¹ then the fluvial flux is 33×10^6 mol yr⁻¹. Assuming that the element behaves conservatively during estuarine mixing and that the average oceanic concentration is 20 pmol kg⁻¹, then the residence time for the element is about 800 yr. As this is shorter than the oceanic turnover time it is important that the value be confirmed in future work.

Crucial to the application of ¹⁰Be in marine geochronology is the degree of uniformity of the ¹⁰Be/⁹Be ratio in the oceanic water column. If tracer equilibrium is achieved during oceanic mixing then changes in the initial uptake of the radioisotope in the sedimentary substrate caused by changes in, for example, composition and accumulation rate can be corrected for directly. The concentration of ¹⁰Be in seawater has been

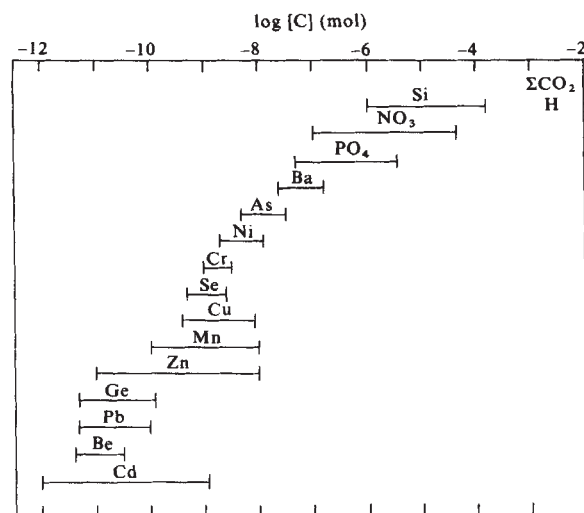


Fig. 3 Published concentration [C] ranges for oceanic trace metals. The nutrients and CO₂ are shown for comparison. Data sources: Ba, ref. 15; As, ref. 16; Cu, Ni, Cd and Zn, ref. 17; Cr, ref. 18; Se, ref. 19; Mn, ref. 20; Ge, ref. 21; Pb, ref. 22.

measured using accelerator techniques^{11,12}. An average value for nutrient-depleted Pacific surface waters (two samples) is $1.2 \pm 0.3 \times 10^{-18}$ mol kg⁻¹ (ref. 11). On the assumption of a ⁹Be concentration of 4 pmol kg⁻¹, the ¹⁰Be/⁹Be ratio becomes $300 \pm 75 \times 10^{-9}$. A ¹⁰Be concentration of $10.2 \pm 2 \times 10^{-18}$ mol kg⁻¹ reported for a single sample from a depth of 4,100 m south-east of San Diego¹², coupled with the deep-water ⁹Be value of 27 pmol kg⁻¹ at MANOP R, give an isotope ratio of $377 \pm 75 \times 10^{-9}$, which is consistent with the surface value.

Yokoyama *et al.*¹³ and Krishnaswami *et al.*¹² have reported isotope ratios in manganese nodules (extrapolated to zero age) of 85×10^{-9} and $114\text{--}1,215 \times 10^{-9}$ with a mean of 255×10^{-9} respectively. Given the present accuracy of the ¹⁰Be measurements ($\pm 20\%$)^{12,14} the overall similarity of the water column and nodule data suggests that the two isotopes are indeed equilibrated in the oceans. Much more work is needed, however, before this important conclusion can be taken as firmly established. In particular, the two isotopes need to be measured on the same water samples. In addition, leaching experiments must be carried out on sediments and nodules to establish that only authigenic beryllium is being analysed rather than an additional component of 'dead' detrital beryllium.

We acknowledge the support and encouragement of our colleagues in the MANOP Program of the NSF/IDOE. We also thank the Office of Naval Research for general support of our analytical program, and Professor K. K. Turekian for helpful discussions.

Received 29 December 1981; accepted 18 February 1982.

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Hindered bedload settling as a model of sand bed planation by water waves

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The interrelationships between fluid flows and the surface forms of underlying movable beds are crucial in interpreting sedimentary structures and in predicting hydraulic drag. Energetic flows can erode all features from a sediment bed, and transition to such a planar bed is important to the processes of sediment transport by waves¹. The following analysis connects bed planation to a threshold effect in hindered settling with increasing concentration of noncohesive sediment moving near the bed. Calculated fluid velocity from the resulting quantitative criterion for this bed transition agrees with extensive laboratory data^{2,3} in oscillatory flows.

The present model of the planation process utilizes simple empirical forms of several of the relationships that are needed. As Reynolds numbers for bed planation are usually large enough for the near-bed flow to be intensely mixed rather than laminar⁴, inertial (non-viscous) asymptotes are suitable for most elements of the model. However, fully-developed turbulent flow is absent in the laboratory situations and Jonsson⁵ has pointed out the persistent empirical accuracy through this transitional range of one result [equation (4)] from laminar flow.

The primary element in the model is an expression⁶ for transport rate of sediment moving near the bed in oscillatory flow:

$$Q/\omega D^2 = [\Phi/10]^{1.5} \quad (1)$$

where Q is average bedload sediment volume per unit time and bed width (moving to and fro colinear with the flow), and $\Phi = [(a\omega)^2/\gamma'gD]$ measures sediment agitation by flow energy. Here ω is flow (wave) frequency, D is median sediment diameter, $a\omega$ is peak near-bed horizontal velocity, g is gravitational acceleration and $\gamma' = (\rho_s/\rho - 1)$, where ρ_s is sediment density and ρ is fluid density. Note that bedload rates reported by Manohar² agree with equation (1) in a range of conditions through the transition from rippled to planar bed⁶.

Conversion of the bedload rate Q into volumetric transport concentration, the ratio of sediment to fluid discharge, requires

Table 1 Test sediments represented in Fig. 1

Label	Sediment description				Maximum ($a\omega^2/g$) included
	γ'	D (mm)	A	n	
Manohar ² spherical grains					
a	1.49	0.235	234	4.2	0.18
b	1.54	0.61	4,590	3.1	0.25
Manohar ² other grains					
c	1.65	0.28	420	3.65	0.22
d	1.63	0.786	9,660	2.3	0.32
e	1.60	1.006	18,900	2.3	0.32
f	0.28	3.17	101,000	2.3	0.09
g	1.60	1.829	108,000	2.3	0.31
h	1.63	1.983	137,000	2.3	0.32
Chan <i>et al.</i> ³ spherical grains					
j	1.65	0.097	18.7	4.6	
k	0.32	0.359	74.4	4.5	
l	1.65	0.254	335	4.05	
Chan <i>et al.</i> ³ other grains					
m	0.97	0.254	79.5	4.4	
n	1.55	0.254	315	3.8	
p	0.97	0.505	625	3.4	
Kennedy and Falcon ¹⁸ spherical grains					
q	0.035	1.0	276	4.1	

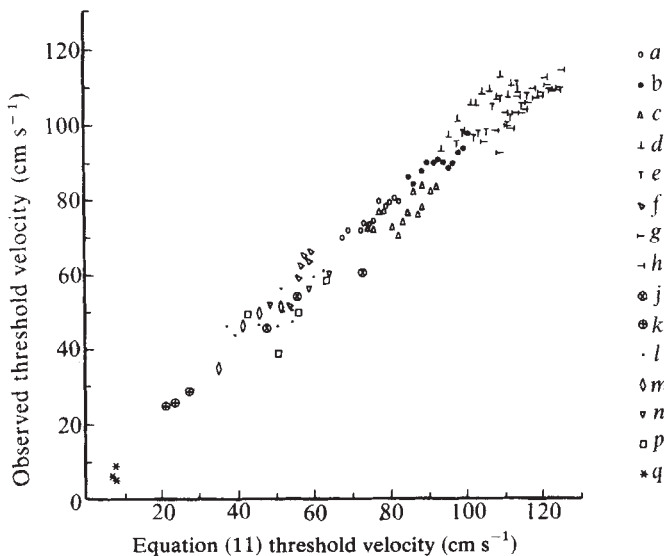


Fig. 1 Threshold velocities for bed planation observed in 120 laboratory tests^{2,3,18} compared with calculations using equation (11). See Table 1 for description of the 15 sediments represented.

division by Λ , a characteristic thickness of the bedload layer, and by Ψ , a characteristic fluid velocity within the layer. Bagnold⁷ reported that the elevation above the bed of this layer's centre (in unidirectional flow) is mD , where

$$m = 1.4(u_*/u_{*0})^{0.6} \quad (2)$$

and the estimate adopted here is $\Lambda = 2mD$. Here u_* is the friction velocity and u_{*0} is critical friction velocity for sediment motion initiation (zero transport). The peak friction velocity is related to peak velocity by⁵

$$u_* = a\omega(f_w/2)^{0.5} \quad (3)$$

where f_w is friction factor, depending on boundary roughness and on the Reynolds number ($a^2\omega/\nu$), ν being fluid viscosity. For the situations of interest with a nearly plane bed, boundary-layer flow is transitional between laminar and turbulent⁸, but the analytical form for a smooth boundary

$$f_w = 2/(a^2\omega/\nu)^{0.5} \quad (4)$$

remains empirically valid although derived for laminar conditions⁵. The proper inertial relation for sand motion initiation in oscillatory flow is⁹

$$(a\omega)_0 = (8\gamma'gD)^{0.5} \quad (5)$$

so that

$$u_{*0} = (a\omega)_0^{0.5}(\nu\omega)^{0.25} = (8\gamma'gD\nu\omega)^{0.25} \quad (6)$$

A suitable velocity profile for estimating Ψ is problematical even in steady transitional (intermittently turbulent) flow¹⁰. The fairly thick bedload layer would appear to be dominated by peak-flow effects here, so the appropriate approximation seems to be an inertial profile with logarithmic variation of velocity, as occurs beyond the viscous sublayer for fluid flow above moderately large Reynolds number¹⁰. The mean horizontal fluid velocity is taken as⁷

$$u = 5.75 u_* \log(30z/k) \quad (7)$$

where z is distance from the boundary and k measures boundary roughness. For a nearly plane bed $k = 2.5D$ is appropriate^{8,11}, and the characteristic velocity is⁷ that at elevation $z = 0.37\Lambda$. With these relationships

$$\Psi = 5.75 (a\omega)^{0.5}(\nu\omega)^{0.25} \log[12.4(u_*/u_{*0})^{0.6}] \quad (8)$$

The postulated link between bed planation and hindered sediment settling is pursued by regarding the right side of equation

(1). The agitation index Φ has¹² an inverse dependence on the square of inertial sediment settling velocity, so that equation (1) indicates an inverse dependence between bedload rate and the cube of settling velocity⁶. The terminal settling velocity, w , of an isolated sphere in still fluid is described¹³ as a variation of (wD/ν) with the buoyancy index $A = (\gamma'gD^3/\nu^2)$, having asymptotic behaviours of viscous settling for $A < 20$ and inertial settling for $A > 10^5$. A homogeneous dispersion of cohesionless spheres settles more slowly than an isolated sphere, with velocity given as¹⁴

$$w_C = w(1 - C)^n \quad (9)$$

where C is volumetric particle concentration, and the exponent n has a smooth variation between $n = 4.6$ for viscous settling and $n = 2.3$ for inertial settling. An isolated natural sediment grain usually settles¹⁵ at slightly smaller velocity than a sphere of the same mass due to increased drag, and inertial settling arises for $A > 10^4$. Effects with natural grain dispersions presumably follow equation (9) if adjustment is made for n between more closely spaced asymptotes.

No data are available for entrained sediment concentrations near a planar bed, but a critical concentration for appreciably hindered settling apparently should be related to grain interactions and the threshold for significant grain-grain collisions has been reported^{16,17} to be C near 0.09 or 0.02. Here $C_{cr} = 0.075$ is adopted; this value is one-eighth of 0.6, the usual maximum possible sediment concentration. Thus the postulated threshold for bed planation becomes

$$(Q/\Lambda\Psi)_{cr} = C_{cr} = \omega D^2(\Phi/10)^{1.5}(1 - C_{cr})^{-3n}(\Lambda\Psi)^{-1} \quad (10)$$

or

$$(a\omega)^{2.2} = 28 \frac{(0.925)^{3n} \nu^{0.25} (\gamma'g)^{1.35} D^{0.35}}{\omega^{0.75}} \log \left[\frac{12.4(a\omega)^{0.3}}{(8\gamma'gD)^{0.15}} \right] \quad (11)$$

With given material characteristics, equation (11) is an implicit relationship for flow conditions at the postulated threshold of bed planation: specifying either flow frequency (ω) or near-bed horizontal flow amplitude (a), the critical value of the other variable is determinable by root-finding. To clarify the dependences of the above threshold, an approximate explicit form is

$$a\omega = 2.8(\gamma'g)^{0.64} D^{0.22} \nu^{0.07} \omega^{-0.35} \quad (12)$$

For the test conditions considered here, equation (12) regularly gives a threshold velocity within a few per cent of that determined by the root of equation (11).

Calculated threshold velocities using equation (11) are compared in Fig. 1 with results from 120 laboratory tests of bed planation with the sediments described in Table 1. Calculations are close to the (very low) near-bed threshold velocities reported for three wave-tank tests conducted by Kennedy and Falcon¹⁸ at transition from rippled to planar bed with lightweight spheres. However, only part of the experimental results from the two major oscillatory-flow studies^{2,3} are presented in Fig. 1 for the following reasons.

Manohar² performed tests by oscillating at fixed amplitudes a sediment tray in still water, and observing the critical frequency for bed planation; analogously, equation (11) has been solved with the specified a to find the threshold ω . Ignoring the independent ω effects, Manohar provided this correlation of results:

$$a\omega = 20.9(\gamma'g)^{0.4} \nu^{0.2} D^{0.2} \quad (13)$$

For accelerated-bed tests, Bagnold¹⁹ reported that general sliding of sediment can occur at large accelerations, depending on ρ_s/ρ and the angle of sediment repose. Consequently a minority of these tests with large $a\omega^2$ has been disregarded, with the cutoff values of $a\omega^2/g$ listed in Table 1; Manohar's tabulated results at small amplitude increments exhibit in each test series a sizeable gap in $a\omega^2$ and $a\omega$ around the stated $a\omega^2/g$. For the remaining 90 tests, with appropriate values of n (Table 1),

agreement is evident between experimental and calculated results, although there is a tendency with some sediments for observed threshold velocity to be $\sim 10\%$ less than that calculated.

Calculations show agreement only with a quite limited segment of results from the other major study. Chan *et al.*³ executed tests by oscillating fluid at fixed frequencies above stationary sediment in a 5-cm diameter pipe, observing critical fluid amplitude for bed planation; equation (11) has been solved by a corresponding procedure. About one-third of the material combinations tested are represented in Fig. 1; these correspond to A values for subaqueous fine and medium quartz sands (although some results for both water and ethanol are shown). More scatter is to be expected here because results have been scaled off a log-log plot for the present use. The agreement of these 27 measurements with calculations by equation (11) is about as ideal as with transition calculations by the following expression, provided as an empirical correlation of all results by Chan *et al.*³:

$$a\omega = 6.6(\gamma'g)^{0.5}\nu^{0.2}D^{0.1}\omega^{-0.2} \quad (14)$$

On the other hand, equation (14) is clearly superior to equation (11) in agreement with the data for other sets of tests with: large D , large ν (glucose) or iron ore. The causes for the present model's failure are not certain, but these cases might be explained by the facts that: equation (4) is inaccurate for relatively large D/a (ref. 8); the small test facility gives²⁰ laminar flow of glucose, making equation (7) inapplicable; and iron ore exhibits cohesion by its magnetism, making equation (9) incorrect. Also, no heavy minerals such as iron ore are represented in the data base⁶ supporting equation (1).

I conclude that the model of bed planation providing equation (11) is consistent with laboratory results for 15 sediments having A values corresponding to quartz sand in water, and segments of the two major data sets^{2,3} show some congruence, whereas these results had appeared disjoint^{3,21}. Note that this analysis has a markedly different viewpoint from that of Bagnold's criterion for planar bed occurrence^{7,22}, and that the latter has been supported in data reviews^{1,23} for unidirectional and oscillatory flows. The present results are established for laboratory transitions to a planar bed from ripples with wavelength governed by a , rather than from bedforms occurring in flows at lower frequencies²¹.

The results reported here, unless otherwise noted, are based on research conducted at the Coastal Engineering Research Center, US Army Corps of Engineers. The findings are not to be construed as an official US Department of the Army position unless so designated by other authorized documents. Permission to publish the present results is appreciated.

Received 7 December 1981; accepted 19 March 1982.

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Acid rain on Bermuda

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Increased acidity of precipitation due to combustion of fossil fuels has been well documented for both the eastern USA¹ and Canada². The SO₂ and NO_x emitted by the burning of coal, natural gas, fuel oil and petrol are oxidized in the atmosphere to sulphuric and nitric acids which subsequently give rise to acid precipitation¹. However, the SO₂ and NO_x emitted, and their oxidation products, are not all removed by atmospheric deposition over the North American continent; a large fraction is advected east out of North America³. In a study between 1 May 1980 and 30 April 1981, we have detected acid precipitation ($pH < 5.6$) on the island of Bermuda, which is $\sim 1,000$ km east of the Atlantic seaboard of the USA. We report here that the acidity of such precipitation is eight times greater on a volume-weighted annual average than rainwater in natural atmospheric equilibrium, and that the acids present are almost wholly sulphuric with a small nitric acid contribution. There is a strong correlation between the presence of these strong acids and the meteorological back trajectory of Bermuda storm systems to the North American continent, which suggests long-range atmospheric transport of acid rain precursors to Bermuda, these having similar anthropogenic origins (that is, from remote fossil fuel combustion) to acid rain precursors on the continent.

Preliminary studies of rainwater in Bermuda from February 1979 to April 1980 suggested the existence of acid rain in Bermuda with a possible seasonal element⁴. To document these observations further, we have sampled all rainfalls in Bermuda from 1 May 1980 at a site at the eastern end of the island, 60 m above sea level, which is thought to be remote from local pollution. We discuss here the data collected up to 30 April 1981.

Samples were collected on an event basis using a wet-only HASL collector⁵ for all months except from late August to mid-September 1980 when the collector malfunctioned. During this period we used a bulk collector manned on an event basis. During the year there were 109 rainfalls for which we measured the pH. In addition, 70 of these rainfalls provided sufficient samples to determine concentrations of all the major anions and cations. Total rainfall during the year was 150.22 cm compared with a long-term average of 146.46 cm (ref. 6). Samples were analysed for pH, strong and total acidity⁷ and all major anions and cations, using standard colorimetric, atomic absorption and ion chromatographic techniques. In all cases the analyses and pH measurements are accurate to better than 10% and ± 0.1 pH units, respectively. The results for all wet-only samples are presented in Table 1.

We have found that precipitation in Bermuda is acidic (Fig. 1): For the entire sampling period, the volume-weighted mean pH of all wet-only samples was 4.74. Gran's titrations⁷ were performed on 29 samples having a $pH < 4.8$. Regression analysis of free acidity, derived from the pH (measured by glass electrode), against strong acidity (determined by Gran's titration) for these 29 samples gave a slope of 0.95 and a regression coefficient, r , of 0.947, indicating that strong acids are responsible for more than 90% of the acidity in Bermuda's rainwater.

The results in Table 1 indicate the presence of excess calcium, potassium, sulphate, nitrate and ammonia over that introduced to the rainwater by sea salts. The calculations of excess are

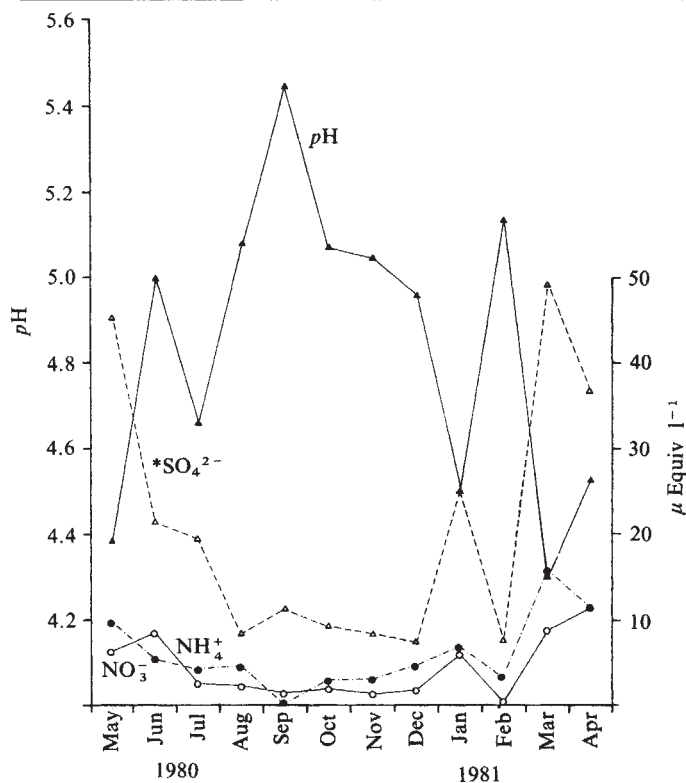


Fig. 1 Volume weighted monthly mean pH (▲) and concentration of NO_3^- (●), SO_4^{2-} (△) and NH_4^+ (○) of rainfall collected in wet-only samples from Bermuda. * Non-sea salt component. The accuracy of each individual analysis is better than 10% and pH measurements are accurate to better than ± 0.1 pH units.

based on sodium, which is assumed to be derived entirely from unfractionated sea salt. The source of the excess calcium and potassium is believed to be clay and rock particulates of either remote aeolian or local limestone origins. The excess sulphate and nitrate in the rainwater is responsible for the acidity. The amount of basic unfractionated sea salt (bicarbonate and boric acid) is insufficient to cause significant neutralization of the acidity. When protons are correlated with non-sea salt sulphate and nitrate concentrations (12 data points per parameter) the slopes are 0.935 ($r = 0.941$) and 0.257 ($r = 0.909$), respectively. This is interpreted to mean that the upper limits of H_2SO_4 and HNO_3 contributions to free acidity are 94% and 26%, respectively.

There are three possible sources of the sulphuric acid in Bermuda rain: locally derived acids, those produced by natural marine processes, or long-range transport of continentally-derived acids. A locally-derived source of the acids is unlikely to be important because: (1) there is no manufacturing industry in Bermuda; (2) the sampling site was 15 km from the island's only power generating plant and during most storms the site was to windward of the plant. (3) Although the sampling site was near the airport, we do not believe that this affected the precipitation acidity because the acidity is primarily due to H_2SO_4 , whereas aircraft exhaust emits mainly NO_x relative to SO_2 and SO_4 (ref. 8). (4) The air mass transit time across the island is very short, giving rapid dispersion and dilution of locally derived pollutants. The meteorology of Bermuda exhibits little or no 'island effect' (ref. 9 and M. J. Nemkosky, personal communication). (5) The observed seasonal pattern described below is incompatible with a local source as power use and air traffic are at a maximum in summer in Bermuda while winds are at a minimum. These factors would produce a summer pH minimum if local sources were important, which is the reverse of the pattern observed.

The next possibility is that of natural marine processes giving rise to acidic gases. The emission of various volatile sulphur compounds from seawater has been reported elsewhere¹⁰. We

do not have a direct measure of the significance of these processes, but using the data of Nguyen *et al.*¹⁰, it is possible to make a rough calculation of their significance; the total potential input from the Atlantic to the west of Bermuda can be compared with the anthropogenic flux of sulphur calculated to leave the USA ($3.9 \times 10^{12} \text{ gS yr}^{-1}$)³. We estimate the sea area to the west of Bermuda that is bounded to the north by the Canadian border and in the south by the southern tip of Florida, to be $\sim 2.5 \times 10^{12} \text{ m}^2$. By scaling down the global ocean estimate¹⁰ of $35 \times 10^{12} \text{ gS yr}^{-1}$, we estimate that the oceanic emission from the sea area to the west of Bermuda could be $\sim 0.2 \times 10^{12} \text{ gS yr}^{-1}$. However, this is probably a maximum value as primary production in this area of the Sargasso Sea is much lower, without upwellings such as those found along the west coast of Africa where many of the measurements of Nguyen *et al.*¹⁰ were made. This figure for sulphur produced by natural marine processes is at least 20-fold lower than the anthropogenic sulphur input calculated by Galloway and Whelpdale³, thus we consider an anthropogenic source for the excess sulphur in Bermuda's rainwater to be the more important. Indeed, aerosol measurements over Bermuda have suggested the presence of anthropogenic sulphate and some trace metals^{11,12}.

In addition, the major weather patterns in the North Atlantic move in an easterly direction, thus transporting the USA air mass towards Bermuda⁹. The meteorology of the Bermuda area of the North Atlantic Ocean is dominated by the semipermanent sub-tropical Azores/Bermuda high. During the summer and autumn, this high pressure can prevent many of the frontal systems originating over North America from reaching Bermuda. During winter, with a weak or absent sub-tropical high, frontal systems pass regularly. This situation becomes firmly established over the islands during December and persists for about 3 months. Late autumn and spring are the transitional seasons. During June to December, pH values are generally high and the excess sulphate, nitrate and ammonia concentrations are relatively low. However, from December to May the reverse is true (Fig. 1). This agrees with the general meteorological pattern described above, although July 1980 and February 1981 represent exceptions. In July an unusually intense storm left the United States coast and reached Bermuda, stalling over the island and producing exceptionally heavy (20 cm over 3 days) and acidic (pH 4.4–4.7) rain which biased heavily the monthly average (Fig. 1). In February 1981, a blocking high pressure was established temporarily, bringing unseasonably mild weather for the first 3 weeks of the month. This North Atlantic meteorological effect is seen in the chemistry of the rain for these months.

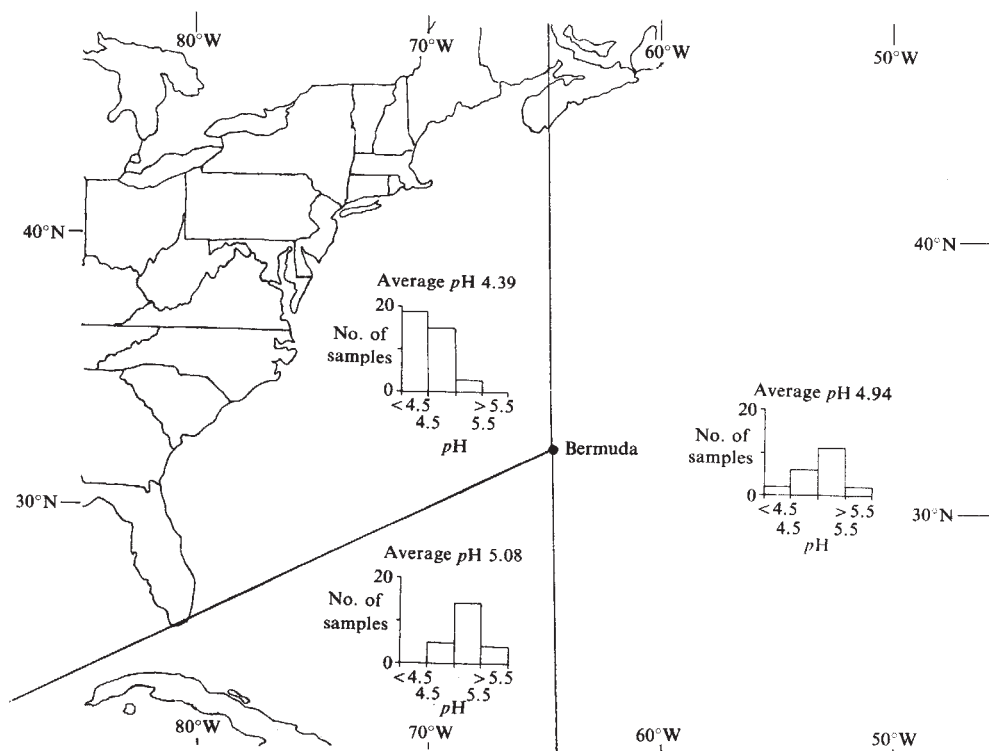
Figure 2 shows the average pH of rainwater associated with air masses arising in each of the three marked sectors as derived

Table 1 Volume weighted mean annual concentrations of major constituents in Bermuda rain from 1 May 1980 to 30 April 1981

Ion	Conc. ($\mu\text{Equiv l}^{-1}$) in all wet-only samples
H^+	21.15
SO_4^{2-}	43.0
NO_3^-	6.31
Cl^-	221
NH_4^+	4.22
K^+	4.77
Mg^{2+}	46.1
Na^+	178
Ca^{2+}	18.0
Total cations	273
Total anions	270
* SO_4^{2-}	21.54
K^{**}	0.99
Mg^{2+*}	5.60
Ca^{2+*}	10.15
* $\text{SO}_4^{2-}/\text{NO}_3^-$	3.40
$\text{NO}_3^-/\text{NH}_4^+$	1.50
Na^+/Cl^-	0.81
* $\text{SO}_4^{2-}/\text{NH}_4^+$	5.10

* Non-sea salt components.

Fig. 2 Map of the north-west Atlantic Ocean showing the three sectors used for ARL back trajectories of Bermuda rain. The average pH of rain originating in each sector is indicated and the distribution of pH values for rain samples from that sector are shown in the histograms.



from ARL back trajectories^{12,13}. Clearly, the rainwater originating over continental North America is markedly more acidic than rainfall originating in the other sectors.

A comparison of the composition of rainfall in Bermuda with recently published data from the eastern United States¹⁴ reveals that in addition to a large increase in sea salt concentrations and a reduction in acidity by a factor of 3 during transit from the US coast, there is an increase in the $^*SO_4/NO_3$ ratio, indicating an increased relative importance for sulphuric acid. There are several possible reasons for the selective long-range transport of sulphuric as opposed to nitric acid rain precursors to the western North Atlantic from North America—these are discussed elsewhere¹⁵.

In addition to demonstrating the existence and causes of acid precipitation on Bermuda, our results suggest that the dispersion and neutralization of acid rain exported from continents may be a slow process. Over the North Atlantic to the west of Bermuda this appears to be due to the absence of suitable neutralizing agents in the troposphere. In view of this finding, we believe that long-range atmospheric transport of acid rain is possible. Indeed there is growing evidence of acid rain on remote areas¹⁶ although the cause of this acidity is often not as clearly defined as for Bermuda.

We thank Susan Boyd for assistance; Commander M. J. Nemkosky Jr and his staff for meteorological information; and Mr A. Atwood and his staff at Bermuda Harbour Radio. This work was supported by the Bermuda Government, British Petroleum Trading Co. Ltd., ESSO Bermuda, US NOAA, EPA, and US DOE. This is contribution no. 891 from the Bermuda Biological Station and contribution no. 1 to the Global Precipitation Chemistry Programme.

Received 30 November 1981; accepted 26 February 1982.

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Radiations and extinctions of plankton in the late Proterozoic and early Cambrian

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The intrinsic palaeobiological importance of planktonic algal microfossils of Proterozoic and early Cambrian age has been widely documented^{1–8}. The evidence supporting the biostratigraphical significance of planktonic microfossils in the correlation of Upper Proterozoic and Lower Cambrian sequences has also been assessed^{1,4–8}. We contend here that both within-flora (α) and total (γ) taxonomic diversity trends for late Proterozoic planktonic microfossils indicate that a distinct radiation of presumed cyst-forming eukaryotic plankters occurred during late Riphean and early Vendian (used here in the sense introduced in refs 1, 8). Following this, a mid- to late Vendian extinction episode reduced observable plankton diversity by some 70%, extirpating most morphologically complex, pre-existing taxa. A second radiation restored high plankton diversity levels, but not until well into the early Cambrian.

Organic-walled fossils of Proterozoic planktonic microorganisms are preserved in a wide variety of sedimentary rocks, ranging from lagoonal mudstones to distal turbiditic shales and greywackes. Microfossil assemblages can be correlated with sedimentary environments, permitting the palaeoecological distributions of both taxa and diversity patterns to be recognized²⁻⁴. The stratigraphical ranges of many late Proterozoic plankters are similarly discernible and from these has emerged a distinctive series of informal assemblage zones that makes possible the confident biostratigraphical subdivision and correlation of Upper Proterozoic sedimentary sequences^{2,4-8}. Because it is possible to observe both diversity patterns within a single time plane and biostratigraphical distributions in successive time intervals, it should be possible to examine planktonic diversity trends through late Proterozoic time. Such analysis reveals both expected patterns of taxonomic diversification and a hitherto unsuspected decrease in taxonomic diversity that took place near the end of the Proterozoic era.

The planktonic nature of Proterozoic microfossils can be inferred on at least four grounds:

(1) The fossils in question tend to be large (10–>500 µm), robust walled, and, often, ornamented vesicles (Fig. 1). Several Proterozoic taxa further exhibit regular median wall splits, polygonal wall openings, or operculate structures, all of which probably represent excystment mechanisms^{2,4,7,8}. In all of these morphological traits, the Proterozoic fossils are closely comparable to Phanerozoic fossil plankton, as well as to the zoosporangia of extant cyst-forming plankters such as prasinophycean green algae⁸.

(2) The conditions of occurrence and environmental distribution of Proterozoic plankters are also quite similar to patterns reported from their Phanerozoic counterparts⁸. Palaeozoic⁹, Mesozoic¹⁰, and modern phytoplankton species tend to have wide facies distributions, but biotas can be resolved into inshore and offshore associations. The same is true for late Proterozoic

plankters⁸. Inshore and, especially, lagoonal biotas are characteristically low diversity assemblages dominated by one or a few taxa, while contemporaneous assemblages from open shelf rocks include a diverse and heterogeneous array of morphologically complex forms^{2,3,7,8}.

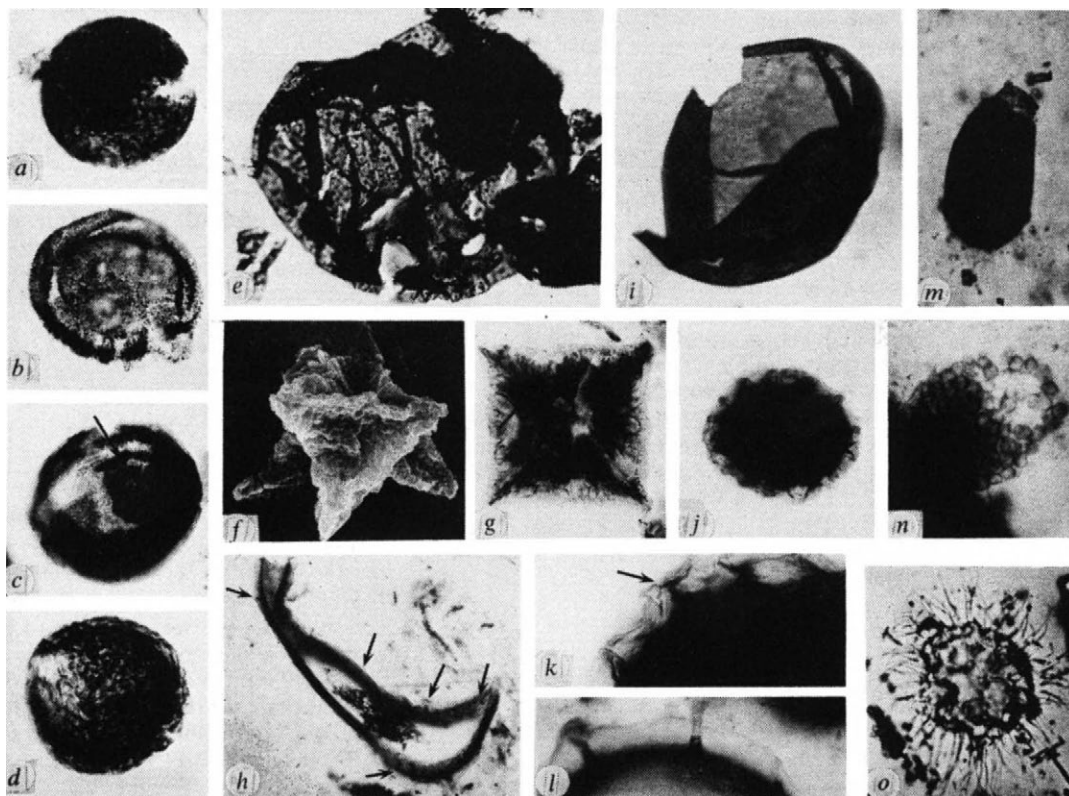
(3) When observable, the distribution of Proterozoic plankters is seen to be independent of the distributions of preserved benthic microbial associations.

(4) As viewed in petrographical thin section, fossils interpreted as planktonic are generally more or less randomly distributed within rock samples, in sharp contrast to known microbial mat builders and other microbenthos which tend to be distributed along bedding.

In general, the morphological and palaeoecological criteria outlined above parallel those used to infer the nektonic or planktonic nature of such extinct Phanerozoic groups as ammonites, conodonts, or graptolites. Although there is room for error in interpretation, we are confident that virtually all of the microfossils included in this study do represent planktonic organisms that inhabited coastal or shelf Proterozoic seas.

The taxonomic literature on Proterozoic planktonic microfossils contains numerous synonymies, descriptions of degradational forms, contaminants, and pseudofossils, and other problems that preclude the simple lifting of species lists from published reports. Therefore, for our analysis, we examined sequences of microfossil assemblages spanning the interval from the beginning of the Upper Riphean¹ to the mid-Upper Cambrian, using only those species whose type specimens have been examined by one of us¹ (G.V.). Successions examined come from East Greenland, northern and southern Norway, Sweden, the Russian Platform, the southern Urals, and Svalbard. Taxonomic diversity was compiled at the specific level (genera and suprageneric taxa are decidedly artificial), species being here defined, as in most other palaeontological applications, as the smallest morphologically definable units that are consistently recognizable and differentiable in

Fig. 1 Representative late Proterozoic and early Cambrian planktonic microfossils. Bar above *o*, 50 µm for *a* and *k*; 25 µm for *b–d*, *g*, *j*, and *m*; 30 µm for *e* and *o*, 20 µm for *f* and *i*, 140 µm for *h*, 70 µm for *l*, 12 µm for *n*. (*a*) *Favosphaeridium favosum* Timofeev, Lower Vendian units of Visingsö Beds, Sweden; (*b*, *c*) *Trachysphaeridium lauffeldi* Vidal, Upper Riphean units of Visingsö Beds, Sweden (arrow in *c* points to apparent operculate excystment structure); (*d*) *T. laminarium* (Timofeev) Vidal, same locality; (*e*) *Kildinella* sp. (*b* of ref. 4), Lower Vendian Ekkerøy Formation, East Finnmark, Norway; (*f*, *g*) SEM and optical views of *Octoedryxium truncatum* (Rudavskaya) Vidal, Lower Vendian units of the Visingsö Beds, Sweden; (*h*) and (*i*) *Trachysphaeridium* sp. (undescribed species) Upper Riphean Hunnberg Formation, Svalbard (arrows in *h* show spines protruding from robust inner wall; *i*, an enlargement of the lower surface of *h*, showing columnar spines and the thin outer membrane they support); (*j*) *K. lophotriata* Jankauskas, Upper Riphean Kwagunt Formation, Arizona (note striate wall structure); (*k*) *Vandalosphaeridium reticulatum* (Vidal) Vidal, Lower Vendian units of Visingsö Beds, Sweden (*k* is a detail of *j*, showing the internal supports for the outer wall of the fossil); (*m*) vase-shaped heterotrophic protist, Upper Riphean Backlundtoppen Formation, Svalbard; (*n*) *Bavlinella faeolata* (Shepeleva) Vidal, Upper Proterozoic Mineral Fork Formation, Utah; (*o*) *Baltisphaeridium ornatum* Volkova, Lower Cambrian Mickwitzia Sandstone, Sweden.



both time and space¹¹. The diversity levels plotted for successive stratigraphical intervals in Fig. 2 represent total (γ) diversity; however, almost all forms examined are cosmopolitan and the within-flora (α) diversity trends for each of our several localities closely mirror the figure presented. Note that while our study centred on the peri-North Atlantic region, sequences in part time-equivalent from as far away as Arizona and Namibia exhibit comparable trends. Further, although individual taxonomic decisions may be disputed by other students of early fossils, we believe that the overall shape of the curve, that is, its qualitative characteristics, is accurate and reflects real events in early plankton evolution.

All but one of the microfossil species used in Fig. 2 (*Bavlinella faveolata*) are undoubtedly eukaryotic, and so Fig. 2 can be viewed as a depiction of the early evolution of nucleated cells. The oldest microfossils that can be interpreted with some confidence as probable eukaryotes are found in ~1,400-Myr old shales of the lower Belt Supergroup, Montana¹². In addition to filamentous cyanobacterial sheaths and thin-walled spheroidal vesicles (possibly envelopes), this microbiota contains what may be the oldest robust-walled acritarchs. Data for the ensuing Middle Riphean interval are sparse, but available information suggests that diversity rose gradually during this period.

As is evident from Fig. 2, Upper Riphean sequences are characterized by the widespread expansion of sphaeromorphic acritarchs displaying a wide variety of sculptural patterns and possibly excystment mechanisms^{7,8}. The succeeding Lower Vendian¹ (perhaps equivalent to the uppermost Riphean Kudashian of the southern Urals) biota is even more diverse. The significance of this expansion is perhaps best understood by analogy to the well known Cretaceous diversification of angiosperm pollen taxa documented by Doyle¹³. Arguments over flowering plant antiquity long centred on the interpretation of scattered and, often, poorly preserved individual plant fossils. Doyle demonstrated that no matter when angiosperms first evolved, the early Cretaceous period marked the evolutionary radiation and ecological rise to dominance of the group. We contend that the late Riphean diversification of marine acritarchs tells a similar story. It is unlikely that this radiation documents the initial appearance of eukaryotic cellular organization. Indeed, most models for eukaryogenesis call for early cells that were wall-less and, hence, virtually unfossilizable. The late Proterozoic expansion does, however, record the initial expansion and rise to ecological importance of presumably cyst-forming eukaryotic plankton, and, thus, the initial radiation of nucleated organisms observable in the fossil record. As far as known, eukaryotes radiated in the late Riphean and early Vendian intervals and, insofar as the fossil record permits us to compare different environments, first established dominance in the planktonic realm¹⁵. By the end of the Riphean era, the preservable plankton biota included vase-shaped heterotrophic protists, as well as algae^{16,17}.

A drastic change in the marine plankton biota occurred in mid-Vendian (Varangerian¹⁸) times, coincident with the widespread Varangerian glacial epoch⁸. Almost 70% of pre-existing taxa disappeared, including almost all of the morphologically complex early Vendian acritarchs and leaving a depauperate biota of simple sphaeromorphs and multi-sphere microfossils interpreted as endosporulating cyanobacteria (*B. faveolata* (Shepeleva) Vidal²). *B. faveolata* is known to have dominated glacially influenced marine environments, probably proliferating in ecologically stressed conditions^{18,19}.

Although the Varangerian plankton record leaves much to be desired because of associated erosional contacts and hiatuses, the reality of the extinctions can be supported on several grounds. (1) Marine Varangerian rocks do exist and contain low diversity assemblages. Such within flora diversity does not depend on outcrop representation in the same way as does total diversity (at least for biotas in which not all forms are cosmopolitan)²⁰. (2) One can argue that observed low Varangerian plankton diversities result from a biased sampling of environ-

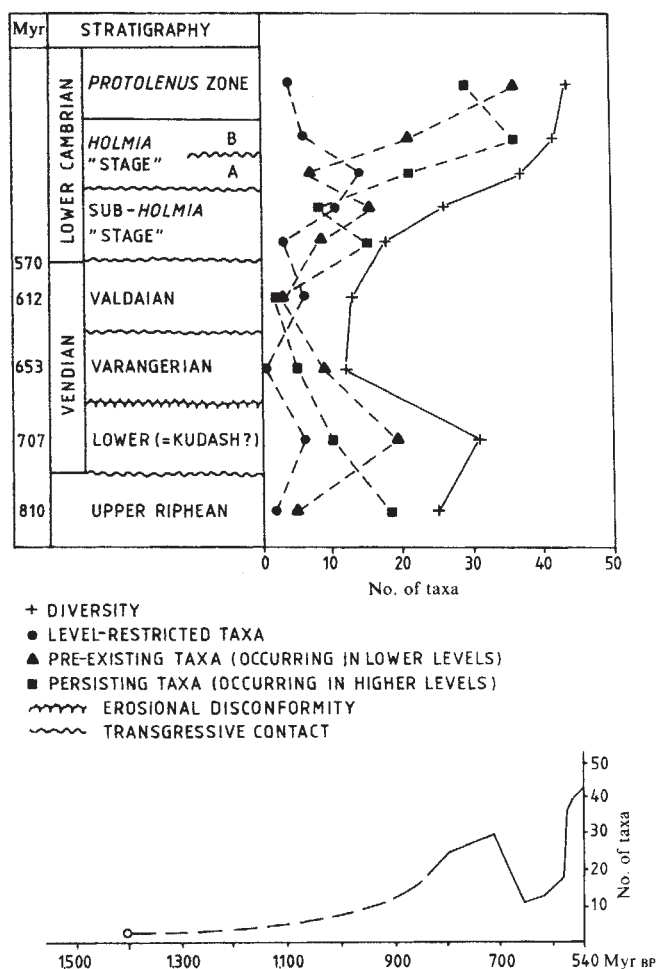


Fig. 2 Diversity changes in the late Proterozoic and early Cambrian plankton record. Upper chart shows details of diversity changes near the Proterozoic-Cambrian (wavy lines between stratigraphical intervals indicate transgressive contacts in Scandinavia; saw-tooth lines indicate erosional disconformities). The lower chart illustrates diversity changes within a broader framework of time. The dotted line is an estimate of the course of eukaryotic plankton diversity from 1,400 Myr BP, the date marking the oldest known probable eukaryotic plankters, to the Upper Riphean (where data become quantitatively reliable). The charts originate from published and unpublished data of continuous Upper Proterozoic-Lower Cambrian sequences in Greenland, Scandinavia (including Svalbard), the Russian Platform, the Urals, and so on, comprising thousands of investigated samples. The microfossil taxa on which the numerical data are based are listed in refs 1, 2, 4-6, 17, 18, 19, 21-23. The Vendian is here used in the sense proposed in refs 1, 8, and comprises pre-Varangerian, Lower Vendian, units in Scandinavia which may perhaps be equivalent with the terminal Riphean (Kudashian) of the southern Urals in the USSR. Definitive proofs for this are, however, missing.

ments—that we are looking at glacially influenced sequences. This is true for the most part; however, immediately superjacent Valdaian (Upper Vendian) strata are equally species poor, and these deposits are widespread and well sampled. Taxa that disappeared at the end of the early Vendian (= Kudashian?) period simply did not reappear, even though non-glacially influenced environments appropriate for their growth and preservation returned. (3) The taxa that disappeared apparently became extinct without issue. Not only do diagnostic acritarchs like *Trachysphaeridium lauffeldi* and *Kildinella lophostriata* (Fig. 1) disappear at the end of the upper Riphean-early Vendian period, but younger acritarch assemblages contain no fossils that seem to belong to the same phylogenetic clade. Thus, there is little doubt that the latest Proterozoic eon witnessed a significant extinction episode in the microplanktonic realm. It is tempting to relate this biological event to the Varangerian glaciation(s) because of the time coincidence; however, any hypothesis relating the two phenomena must be speculative.

Plankton diversity rebounded sharply in early Cambrian times (Fig. 2), with the radiation of spinose forms attributed to the Palaeozoic acritarch genus *Baltisphaeridium* and other new and morphologically complex acritarch forms. When it occurred, the Cambrian radiation was remarkably rapid; however, note that the most comprehensive radiation does not coincide in time with the beginning of the Cambrian period²¹. Plankton diversity underwent a slow increase throughout the Sub-Holmia 'stage'^{22,23} of the lower Cambrian system.

We conclude that the fossil record of early eukaryotic plankton has a distinctive and not entirely predictable shape. Late Riphean and early Vendian microplankton assemblages indicate a dramatic morphological (taxonomic) diversification that represents the first recognizable radiation of eukaryotic algae and documents their rapid rise to dominance in coastal planktonic habitats. An equally striking drop in diversity near the end of the Proterozoic era is interpreted as the first recognizable episode of widespread extinction. More data, particularly from sedimentary sequences outside the peri-North Atlantic area will be required to test and refine the arguments presented here.

This work was supported by grants from the Swedish Natural Science Research Council (N.F.R.) and the Knut och Alice Wallenberg Stiftelsen (G.V.) and NSF grant DEB 80-04290 (A.H.K.).

Received 7 January; accepted 17 March 1982.

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Table 1 Mating and song sharing in the great tit

No. of songs shared with female's father			
<i>a</i>			
	0	1	>1
Husbands	6	11	0
Other males	242	157	45
<i>b</i>			
	0	1	>1
Husbands	6	11	0
Other males	84	50	10
<i>c</i> Degree of difference between father and husband*			
	'Similar'	'Intermediate'	'Different'
Observed (husbands)	3	5	1
Expected (other males)	3.15	1.8	4.05

a, The number of females mated to males sharing 0, 1 and more than 1 song with the female's father. This distribution was compared with one obtained by counting all the males (except for fathers) in Marley Wood in the year in which the female in question bred. The table shows overall heterogeneity ($\chi^2_{2d.f.} = 6.8$, $P < 0.05$), and a comparison of 'one song shared' versus 'no songs' or 'more than one song' is significantly different from expected ($\chi^2_{1d.f.} = 6.09$, $P < 0.02$). *b*, A similar analysis to that in *a*, but considering only males that nested between 201 and 400 m from the female's birth site. The table shows overall heterogeneity ($\chi^2_{2d.f.} = 6.6$, $P < 0.05$). Comparison of 'one song shared' versus 'no songs or more than one' $\chi^2_{1d.f.} = 5.82$, $P < 0.02$. *c*, Of the 17 husbands analysed above, 11 shared one song with the female's father. In nine of these cases other males in the husband's year also shared the song with the father. For these nine songs it was possible to compare the difference in detailed song structure between father and husband with that between father and other males. Husbands fell into the category 'intermediate difference' slightly more often than expected ($P = 0.06$, Fisher exact test). It is reasonable to test the table directly for an excess of 'intermediate' mates because *b* predicts this.

* (1) Take six measures of song structure for all males (including husband) that share the song type in question with female's father. (2) Rank males in order of similarity to father on each measure. (3) Divide rank orders in three equal-sized categories: 'similar', 'intermediate', 'different'. (4) For each male determine which category is commonest. (Ties: share tied categories.) Use this as estimate of overall similarity. (5) Compare observed distribution of similarity between husbands and fathers with that expected based on all other males.

were individually ringed throughout the study. The recordings were made before and during the breeding season (March–May) by visiting all the territorial males several times to record both spontaneous song and song produced in response to playback tapes. Each male had a repertoire of one to six song types ($\bar{x} = 2.98$, $n = 152$). We classified the 454 songs recorded into 41 categories by inspecting narrow band sonagrams. Great tit song is a loud vocalization consisting of rhythmically repeated units (phrases) which are uttered in bursts with intervals of silence between. The major criteria for classifying songs were as follows³: (1) number of notes per phrase, which varies over 1–11; (2) type of note (pure or modulated); (3) frequency relationship of notes (for example, in a song with two note types, one pure, one modulated, the pure note could be of higher or lower frequency than the modulated; these two possibilities would count as two different song types); (4) duration of song phrase. We placed less emphasis on variation in duration of notes and modulation rate within notes. When P.K.M. and J.R.K. classified songs independently they obtained 85–95% agreement. The song types about which we did not agree were usually rare ones sung by only one bird in a year and we assigned these to new categories.

Our analysis is based on 17 females for which we knew the repertoires of both husband and father. We compared the songs of the 17 'pairs' of female's fathers and mates in two ways: first by looking at the number of song types shared, and second by

Mating and song types in the great tit

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We report here the first field investigation of the relationship between the songs experienced by female songbirds early in life and those produced by their mates. Our 6-yr study of the great tit (*Parus major*) showed that females in a ringed population tended to be mated with males singing slightly 'unfamiliar' songs, where 'familiar' songs are defined as those sung by the female's father. This result parallels recent findings on sexual imprinting in birds^{1,2}.

We recorded the songs of all the breeding male great tits (between 18 and 33 birds in a year) in Marley Wood, Oxford, during 1975–80. Most of the breeding adults and all their young

examining the detailed structure of any songs that were sung by both males.

Table 1a shows that females are more likely than expected by chance to be mated with males that share one song type with the father. There is also a slight but nonsignificant suggestion that the 17 females avoid mating with males possessing 'rare' songs (those used by four or fewer males in a year). The observed number of females mated to a male with more than one rare song was 1 out of 17, while the expected number was 3.55 out of 17 ($P \sim 0.1$ Fisher exact test). Rare songs tend to be sung by immigrant males from outside the wood³. The females were all born within the wood, hence the observed trend suggests avoidance of mating with males from other populations. This conclusion might be spurious if we have overestimated the availability of unpaired immigrant birds, as might happen, for example, if immigrant birds (mostly young ones) arrive already paired. However, young birds disperse in July–August⁴ and usually do not pair until September–February⁵, which suggests that any non-randomness of pairing arises after, not before, birds immigrate.

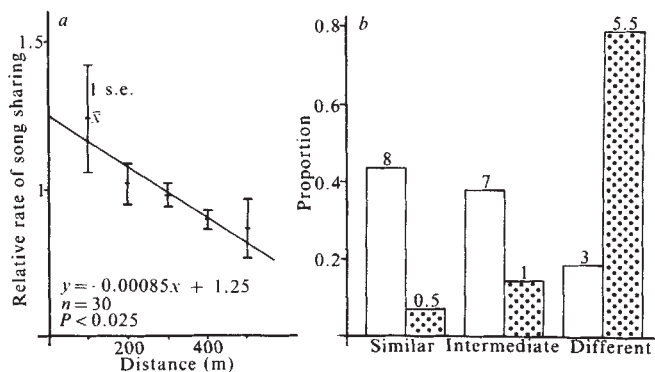


Fig. 1 a, The correlation between the rate of song sharing $[(N/R)]$, where N = number of songs shared and R = repertoire size of the reference male] by males and the distance between their territories. There are five distance categories and we measured the rate of song sharing in each category for 6 yr (therefore $n = 5 \times 6 = 30$). The results are expressed as rates of song sharing relative to the mean for each year in order to eliminate between-year differences in mean rate. The mean number of songs shared between neighbours (distance category 1) is 0.65 songs. b, The similarity of songs shared by fathers and males within the Marley population (open histograms), and fathers and males outside Marley Wood (shaded). The height of the histograms indicates the number of males in each similarity category (see text for calculation) as a proportion of all Marley males ($n = 18$) or all non-Marley males ($n = 7$). Actual numbers are given above each histogram, that is, eight Marley males share a song type which is the same as the father's and is similar in detailed structure. Only two song types were found within and outside Marley; the results for these have been combined.

Table 1 computes the expected distribution by counting the number of males sharing 0, 1 and >1 songs with the father. In this calculation we pooled all the males in Marley Wood during the year in which the female in question bred. This assumes as a null hypothesis that a female is equally likely to mate with any male in the wood. However, it is known that females undertake dispersal from their natal site before breeding and are hence unlikely to mate with males nesting very close to the father's territory. Furthermore, the rate of song sharing between males is correlated with distance between their territories (Fig. 1a). We therefore refined our null hypothesis by calculating the expected distribution using only males living between 201 and 400 m from the females' birth site. This corresponds to the average dispersal distance of three territories for females calculated by Greenwood *et al.*⁶. Table 1b shows that females are less likely than expected to mate with males sharing 0 or >1 song with the father. However, the slight

tendency to avoid husbands with rare songs disappears when dispersal is taken into account (the revised expected number is 0 out of 17, the observed is out of 17).

By correcting for dispersal distance, we have tried to eliminate the possibility that our results could be explained solely as a by-product of dispersal distance. This does not, however, show that song and dispersal are unrelated: for example, young birds could be sensitive to the changing frequency of song types with distance (Fig. 1a) in choosing where to settle.

Table 1b can be considered in two parts in order to assess the contribution of avoidance of similar and dissimilar repertoires to the final result. Comparison of columns 2 and 3 of Table 1b shows only a slight suggestion of avoidance of males with very similar repertoires to the father ($\chi^2_{d.f.} = 2.13$) and a similar comparison of columns 1 and 2 shows that significantly fewer than expected husbands occur in column 1 ($P < 0.05$, $\chi^2_{d.f.} = 4.7$). Therefore, both effects contribute to the overall level of significance, but avoidance of dissimilar repertoires has a greater effect.

Table 1c shows that the bias in favour of 'intermediate degree of difference' shown above for song types also applies to detailed song structure. For 9 of the 11 husbands which shared a song type with the female's father we could compare the degree of difference between husband and father with that between fathers and other males in the population (see Table 1 legend for details of the method). Slightly more females than expected were mated to males of intermediate difference. As in Table 1b, we corrected for dispersal distance in constructing our null hypothesis, as the degree of similarity of detailed structure varies with distance between territories¹⁷ (Fig. 1b).

Thus, our results are best explained by the idea that females prefer a balance between similarity and dissimilarity. The trends we have observed could be summarized as 'prefer males that share one song type with father, prefer shared song to be slightly different from father's'. A parallel finding for plumage patterns has been found in experiments on sexual imprinting in quail^{12,7}. This has been interpreted as indicating that females might strike an optimal balance between inbreeding and outbreeding, an effect described in plants⁸. Avoidance of inbreeding^{9,10} (which is known to be disadvantageous in great tits¹¹) and outbreeding¹² has previously been suggested as a possible consequence of song learning in birds. Although our results are consistent with the predictions of optimal outbreeding, we must still test the three main assumptions of the hypothesis: (1) Females learn paternal songs. (2) Song types influence mate choice in the great tit, as found in other species^{13,14}. (Song seems to be important in mate attraction in the great tit because males whose mates have been removed sing more than paired males at the same time of year¹⁵.) (3) There is a cost of outbreeding between populations, as has been shown in some other species^{16,18}.

We acknowledge the SRC for financial support and thank Pat Bateson, Peter Slater, Paul Sherman, Paul Harvey and Chris Perrins for their comments on the manuscript and Andy Dobson, Alan Grafen and Michael Bulmer for statistical advice.

Received 19 November 1981; accepted 23 March 1982.

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Male *Eufriesia purpurata*, a DDT-collecting euglossine bee in Brazil

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While studying the ecology of the malaria vector *Anopheles (Nyssorhynchus) darlingi* Root along the Ituxi River, Amazonas, Brazil, we observed aggregates of bees on the walls of houses that were routinely sprayed with DDT. Several bees collected from DDT-treated house walls in August 1978 were identified as male specimens of *Eufriesia purpurata* (Moscary) of the tribe Euglossini (Hymenoptera: Apoidea). (The bees were identified as *Euplusia purpurata* by Anthony Raw, Laboratorio de Ecologia, Universidade de Brasilia. The change of generic name from *Euplusia* to *Eufriesia* is based on ref. 1.) These bees were well known to the local residents as the insects that eat DDT and we present here the first documentation that they (1) are attracted to DDT, (2) actively collect large quantities of DDT from treated house walls and (3) suffer no apparent insecticidal effects. We also found that the frequency of house visiting is most intense during July to September. Most bees arrive at houses before 12.00 h, remain 2–3 h and return on subsequent days to collect more DDT. Noise produced by bees as they collected DDT was a notable disturbance to 76% of 21 families interviewed along the Ituxi River.

The euglossine bees have been extensively studied due to their important role as plant pollinators. Both males and females provide valuable pollination services, but a more specific relationship has been found for the males in their pursuit of certain flower odours, and many flowers only attract a single bee species that affects pollination^{2,3}. The attractants of male euglossine bees are characteristically aromatics and monoterpenes³.

Five bees collected in 1978 were analysed for DDT residues in accordance with analytical procedures recommended by the United States Environmental Protection Agency⁴. The head, thorax, abdomen and fore-, mid- and hindlegs were processed separately for each specimen (wings were discarded). The body parts were weighed, before DDT extraction, to obtain concentrations of insecticide in parts per million (p.p.m. or µg per g) by body weight.

The sum of the DDT isomers expressed as DDTR, converted to p.p.m. by dry weight, revealed exceptionally high concentrations of DDT (Table 1). Highest concentrations were consistently found in extracts of the hindlegs, indicating that DDT was stored in the hind tibial pouch. This finding was consistent with observations on the storage site of other attractants collected by euglossine bees³.

During investigations made from September to October 1979, the attractancy of DDT to males of *E. purpurata* was demonstrated by exposing two treated boards for each of the major components of the standard DDT wettable powder formulation. The standard DDT powder consisted of 75% DDT, talc and a wetting agent and was sprayed on house walls as a suspension in water. A common house construction material, slats from açai palm, was used for the 22.5 × 9.0 cm test boards. The surface of two boards was wetted for each of the following treatments; (1) three times with 625 p.p.m. DDT (technical grade) in methanol solution; (2) suspension of talc in water; (3) water; and (4) methanol. The specific wetting agent could not be identified and was not included in the test; as it was a minor component of the spray formulation, we decided that the compound should be identified and tested only if there was no apparent DDT attractancy. The treatments described above provided eight boards which were exposed simultaneously to populations of *E. purpurata*.

Treatments were exposed in random block design (two rows of four boards each) on a tree 0.5 m above the ground. Bees were common around the tree because an ant nest at the tree base had been sprayed with DDT in April 1979. Treated boards were randomly assigned to eight fixed positions and were relocated seven times. Visual counts of bees on the boards were made on 3 days. Eighty-three *E. purpurata* landed on the DDT-treated boards, as determined by 23 separate counts, and no bees landed on any other boards. Analyses for DDT surface residues on the treated and untreated boards revealed DDTR concentrations of 1,236 and <1.0 p.p.m., respectively. That the concentrations on treated boards were lower than expected was probably due to leaching of some DDT crystals by a light rain on the last day of observations. These test results demonstrated the specific attractancy of DDT for *E. purpurata* males.

The daily house-frequenting activities of male *E. purpurata* were also studied, either by conducting hourly counts of bees by location in the house or by collecting all bees as they arrived at the house. Bees were collected using aerial nets and numbers captured were recorded at 15-min intervals. Count and collection data were obtained for a 5-day series with hourly counts performed on days 1 and 3. Continuous collections were conducted on days 2, 4 and 5.

The hourly counts produced a mean of 397 bees per day compared with a mean of 135 bees per day captured during 3 days of continuous collections. The collection data indicated that most of the bees that visited the house on a particular day first arrived before 12.00 h. Studies of in-house distributions of bees and insecticide residues revealed that ~78.6% of the bees were located on the inner walls of the house during the 2 days of hourly counts, the remaining 21.4% being observed on the thatch ceiling, exterior walls and wood superstructure of the house. Wall and ceiling samples were collected from four houses to document the within-house distribution of DDT residues. Average DDTR concentrations in p.p.m. (range limits in parentheses) were as follows: upper ceiling, 152 (36–413); lower ceiling, 5,892 (10–9,225); interior wall, 102,101 (40, 670–170,670); exterior wall, 2,120 (35–8,110). Clearly, the within-house site of highest DDTR concentrations corresponded with the site of most intense bee activity.

Twenty-eight bees from these studies were analysed for DDTR concentrations. The geometric mean DDTR concentrations in bees collected immediately on arrival at the house, specimens marked while collecting DDT and captured 24 h later, and specimens marked in the morning and captured in the afternoon, were 1,197, 4,809 and 2,152 p.p.m., respectively. The DDTR concentrations found in bees captured early in the morning, before they could alight on the DDT-treated walls, indicated that many specimens had collected DDT on a preceding day and survived through the night. Furthermore, 3 of about 50 bees that had been marked with an acrylic paint on day 3 were recaptured on day 4.

In July–August 1980, studies were conducted with mark-capture and bioassay methods to define further the effects of

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DDT on bee longevity. The bees were individually marked with USR fluorescent pigment 1953 as they collected DDT from the house walls and were identified with a Blak-Ray, ULV:56, long-wave UV lamp on subsequent capture. This procedure was used in a 6-day series of observations to confirm previous findings on the diurnal activity patterns. The bees were counted hourly and 103 specimens were marked on day 1. Continuous collections of bees were made on day 2; this was followed by a repeat of the first day activities on day 3, when an additional 108 bees were marked. Continuous collections were again made on days 4, 5 and 6. Seventy (33%) marked bees were captured, with 20, 19, 16 and 15 being captured on days 2, 4, 5 and 6, respectively. These results clearly indicate that the bees survived the exposure to DDT and returned on subsequent days to collect DDT from sprayed houses.

Results from this 6-day series of observations on the diurnal activity cycles were in general agreement with the 1979 studies. A daily mean of 188.5 bees were recorded during 2 days of hourly counts whereas the mean number captured per day was 89. The main difference was that proportionately more euglossine bees were captured in the afternoon during the 1980 studies; for example, 60% of the total was captured before 12.00 h in 1980 compared with 92% in 1979. Count and

in five specimens of *E. purpurata* was 2,039 µg per bee (Table 1).

Head of household interviews were conducted in July 1980 for 21 families along the Ituxi River. All individuals knew of the bees and 71% had bees in their houses at the time of the interviews. In addition, 100% responded that there had been no house-visiting bees until the malaria control spray programme was begun. There was unanimous agreement on the time of year that the bees appeared in significant abundance—July–September. Noise produced by bee activity reportedly was not disturbing to 5% of those interviewed, more or less disturbing to 19% and was a notable disturbance to 76% of the families interviewed. Fortunately, male euglossine bees are stingless.

Euglossine bees have been reported to frequent houses in other areas of the Amazon Basin, and have also been observed brushing on house walls near Iquitos, Peru (R. L. Dressler, personal communication). That this is a widespread phenomenon was verified by a recent report on sightings of *E. purpurata* in houses in many areas in the state of Para, Brazil⁶.

Simple summary statistics suggest that populations of *E. purpurata* remove significant amounts of DDT from sprayed houses. An average of 112 bees per day were collected from a DDT-treated house during studies in 1979–80. If each bee removed 2,459 µg of DDT (value = average dry weight of bee × average DDTR in µg per g = 0.0982 × 25,044.6), then a total of 24.8 g of DDT, theoretically, could be removed during 3 months of intense bee activity. This would be equivalent to stripping the DDT from 12 m² of wall surface. However, as individual bees make repeated visits to houses, we conclude that our estimate for numbers of bees per day is an overestimate and, thus, they probably do not remove a sufficient amount of DDT to have an adverse impact on the malaria control effort.

Observations reported here demonstrate that male *E. purpurata* are attracted by the odour of DDT, which stimulates their behaviour of brushing the insecticide from walls for storage in a pouch of the hind tibia. The reasons for this behaviour are unknown but it may be that DDT or some aspect of its structural conformation is mistaken by the male bees for an odour substance which they normally collect in nature. Such substances could serve as territorial markers or they may be metabolically altered and secreted as sex attractants^{3,7,8}. More recent research on the fate of odour substances provides additional evidence that collected substances are metabolized (or altered) by the male bees (N. H. Williams, personal communication). Clearly, many aspects of euglossine biology remain to be clarified.

We thank Professor Aluizio R. Prata, Myron G. Radke and Willis A. Reid Jr for their help and encouragement, Dr Ronald Ward for reading the manuscript and Wilma Rich for typing. This work was supported by the Brazilian Conselho Nacional de Pesquisas and research contract DAMD 17-79-G-9450 from the US Army Medical Research and Development Command, Office of the Surgeon General, Ft Detrick, Frederick, Maryland 21701. The opinions contained herein are those of the authors and should not be construed as official or reflecting the views of the Department of the Army.

Received 20 November 1981; accepted 16 March 1982.

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Table 1 Residue levels of DDTR in male *Eufriesia purpurata* collected from sprayed houses along the Ituxi River, Amazonas, Brazil

Bee no.	Residue levels of DDTR (p.p.m. or µg per g)						Total p.p.m. (µg per g) per bee
	Fore-legs	Mid-legs	Hind-legs	Head	Thorax	Abdomen	
1	452	930	10,526	124	30	119	12,181
2	1,267	4,245	29,083	359	60	203	35,227
3	2,465	6,222	11,203	839	154	121	21,004
4	2,769	1,685	10,631		99		>15,184
5	10,998	3,706	25,026	1,326	399	173	41,627

collection data were also used to calculate crude estimates of time that the average bee remained in the house by dividing the mean number collected in the house per day by the mean daily number reported in hourly counts—2.9 and 2.1 h for studies in 1979 and 1980, respectively. These estimates clearly indicated that individual bees actively collected DDT for a considerable length of time.

The bioassay test consisted of comparing mortalities in bee populations that had been exposed to DDT with unexposed populations. In 1979 we discovered that *E. purpurata* males could be attracted to DDT-treated sheets in the forest. Therefore, at a site ~5 km from human habitations, two screen-encased DDT-treated sheets were used to attract bees, which were captured and subsequently used as normal controls in a bioassay test. Twenty-five bees captured from house walls and 18 captured in the forest were placed individually in tubes, provided with cotton pads soaked with 10% sucrose solution and observed for mortality. All bees were held in a Styrofoam ice chest to maintain a high humidity and protect against temperature extremes. After 54 h, 22% of controls had died compared with only 8% mortality among specimens collected from the DDT-treated walls. When this test was repeated in October 1980, the 48-h mortalities were 20 and 28% for the control and test populations, respectively. Our bioassay test results, plus the mark-capture data, gave no indication that the *E. purpurata* males were in any way harmed by high concentrations of DDT. In addition, we have made observations for many days on hundreds of bees as they collected DDT and no specimens demonstrated insecticidal effects (for example, loss of orientation, weakness, paroxysms). This is surprising because the average LD₅₀ of *p,p'*-DDT for honeybees is 6 µg per bee⁵. In contrast, the average concentration for this isomer

Erythrocytes deficient in glycophorin resist invasion by the malarial parasite *Plasmodium falciparum*

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It has been suggested that one of the main factors which determines host susceptibility to different malarial parasites is the interaction of their invasive forms, merozoites, with specific receptors on the red cell membrane¹⁻³. Thus the Duffy blood group determinants may be involved in the entry of *Plasmodium vivax* but not of *Plasmodium falciparum* into human red cells. When analysing red cells deficient in various blood group antigens, Miller *et al.* noted³ that two samples of En(a-) cells showed a reduction of invasion by *P. falciparum*⁴. These cells lack both the major transmembrane sialoglycoprotein, glycophorin A (or MN glycoprotein), and the independently segregating Wright^b (Wr^b) antigen and also show increased glycosylation of band 3, the major membrane-penetrating glycoprotein. Despite this, En(a-) individuals are haematologically normal⁵. We have now confirmed that En(a-) cells obtained fresh from three En(a-) individuals are indeed relatively resistant to invasion by *P. falciparum* although they are able to support parasite development. Our results also indicate that these cells may be a useful model in the search for a putative receptor for *P. falciparum*.

We have compared the rates of invasion by *P. falciparum* of normal and En(a-) cells obtained from the British⁶ (MEP), Finnish¹⁰ (GW) and Canadian¹¹ (RL) homozygous En(a-) individuals using an *in vitro* culture system⁶⁻⁸ (Table 1). The numbers of parasites found in the En(a-) cells were significantly less than in the En(a-) controls using either the continuous asynchronous culture system⁶ (incubation period 72 h) or the synchronized short-term (12-18 h) microtissue culture tray system⁸. The fact that parasite numbers are smaller in the longer-term cultures with cells from MEP is probably explained by some parasites being able to go through more than one schizogony, thus amplifying the differences between the En(a-) and En(a+) cells.

Because the reduction of invasion into En(a-) cells was relative rather than absolute, it was particularly important to control for contamination by En(a+) cells unavoidably present in the infected blood. This was achieved by using fetal haemoglobin (HbF) as a marker of En(a+) cells in the parasite culture. By using a modification of the acid elution technique¹² to stain for HbF, to exclude En(a+) cells from parasite counts¹³, we confirmed that there was some invasion of En(a-) cells but much less than of En(a+) cells. The more marked reduction in parasite numbers in GW and RL compared with MEP is surprising because whereas MEP cells, in addition to being deficient in glycophorin A, are also partially deficient in glycophorin B¹⁴, a glycoprotein sharing considerable homology with glycophorin A¹⁵, the cells of GW and RL lack only glycophorin A.

Once inside the En(a-) cells, parasites were able to develop as successfully as in En(a+) cells (Table 2). In these experiments also, HbF was used as a marker to control for contaminating En(a+) cells from the infected blood. These results indicate that defective invasion rather than development is the mechanism whereby parasites fail to interact with En(a-) cells.

Because of previous sensitization by transfusion or during pregnancy, the sera of En(a-) individuals usually contain anti-En^a antibodies of varying titres. Just as En(a-) cells have several membrane alterations, anti-En^a sera contain antibodies with at least three specificities—against the portions of the glycophorin A molecule which are respectively sensitive and insensitive to trypsin and against the Wr^b antigen⁵. The effect of anti-En^a

Table 1 Invasion of En(a+) and En(a-) cells by *P. falciparum*

Test cells	Incubation time (h)	En(a+)	Parasites per 100 red cells	
			En(a-)	En(a-)/En(a+)
MEP ₁	72	8.4	1.4	0.17
		6.2	2.0	0.32
		4.9	1.9	0.39
		Mean 0.29 ± 0.06		
MEP ₂	12-18	7.5	3.2	0.43
		12.0	5.0	0.43
		10.3	5.9	0.57
		Mean 0.48 ± 0.05		
GW	12-18	13.4	1.0	0.07
		7.2	0.6	0.08
		Mean 0.08 ± 0.07		
RL	12-18	6.7	0.9 (1.3)	0.13
		9.3	1.4 (2.1)	0.15
		7.5	1.1 (2.2)	0.15
		Mean 0.14 ± 0.01		

The Uganda-Palo Alto strain of *P. falciparum* used was maintained in continuous culture using previously described methods⁶⁻⁸. In the 72-h cultures, cells containing asynchronous parasites were mixed in a 1:10 dilution with uninfected En(a+) or En(a-) cells and grown in Petri dishes with changes of medium every 12 h. In all cases, the cells, if not used immediately, were stored at 4°C in citrate/phosphate/dextrose (CPD). After 72 h, samples from the cultures were smeared and stained to count the number of parasites (all stages) present. At least 1,000 cells were counted. The starting parasite count in these experiments was ~1%. Parasite growth for the short-term invasion assay was synchronized using a 5% sorbitol solution²⁰. Schizonts were concentrated with a 3% gelatin solution (Plasmagel)²¹ and mixed with uninfected cells at a dilution of 1:6-1:20 to obtain a 1.5% cell suspension with 2-5% of the cells containing schizonts; 200 µl of the mixture were pipetted into each well of a microtissue culture plate, incubated for 12-18 h at 37°C in a mixture of 5% O₂/7% CO₂/88% N₂ and then smears were made and stained. Parasite numbers were assessed by counting the number of ring forms in at least 1,000 cells. The relative multiplication in the En(a-) cells was calculated as a proportion of the controls ± s.e.m. Cells from En(a-) individuals were obtained on two occasions from the Sheffield Blood Transfusion Service, UK (MEP), from the Red Cross Blood Transfusion Service, Helsinki (GW) and from the Red Cross Blood Transfusion Service, Montreal (RL). These and control En(a+) bloods taken at the same time were collected in CPD or acid/citrate/dextrose (ACD). (In the experiments with the En(a-) cells of RL, infected blood came from cultures containing fetal haemoglobin (HbF). Thus by staining smears using a modification of the acid elution technique¹² the counting of parasites in the HbF-containing En(a+) cells could be excluded. Figures in parentheses show parasite counts in all cells (adult and HbF-containing).

Table 2 Development of *P. falciparum* in En(a-) cells

Invasion Parasites per 100 red cells (En(a-)/En(a+))	Development Schizonts per 100 singly infected cells	
	En(a+)	En(a-)/En(a+)
0.13*	79	78
0.15*	80	79
0.15*	80	82
0.35	68	70
0.32	67	70
0.16	70	77
0.42	71	79
Mean	0.24 ± 0.04	1.04 ± 0.02

The methods used were as for Table 1. The cells used were from the Canadian En(a-) individual (RL). The medium in the microtissue culture wells was changed at ~15 and 40 h. Development was assessed at 48 h by counting the number of parasites in 100 singly infected cells that had matured to the schizont stage (that is, a large parasite with the chromatin clearly divided into at least two distinct entities).

* In these experiments infected blood came from cultures containing HbF.

Table 3 Invasion of En(a+) cells by *P. falciparum* in the presence of anti-En^a serum

Serum	Titre (reciprocal)	ABS	Parasites per 100 red cells		<u>Unabsorbed</u> Absorbed
			Anti-En ^a (unabsorbed)	Anti-En ^a (absorbed)	
GW ₁	64	9.2	1.7	Not tested	
		5.6	0.6	5.9	0.10
		8.2	1.1	9.3	0.12
		7.4	1.0	7.9	0.13
		3.8	0.6	3.9	0.15
					Mean 0.13 ± 0.07
GW ₂	8	10.1*	4.0	12.7	0.31
		11.5	6.2	14.4	0.43
		9.2*	4.7	10.6	0.44
		11.6	5.0	9.1	0.55
		17.4	12.2	16.0	0.76
					Mean 0.50 ± 0.08
MEP	8	16.1	6.4	18.9	0.34
		7.7	3.5	9.4	0.37
		9.2*	4.4	9.5	0.46
		11.2	5.6	11.0	0.51
		7.6	4.6	8.2	0.56
		10.1*	5.4	9.5	0.59
		17.4	8.7	14.4	0.60
					Mean 0.49 ± 0.04

The methods used were as for Table 1 except that 50 µl of the medium per well was substituted with 50 µl of the serum to be tested²². Group O-positive cells were used in all experiments. Sera from the En(a-) individuals were obtained on several occasions. The first example from GW(GW₁) contained sodium azide added as a preservative. As azide is toxic to parasites, this serum was dialysed with parallel AB serum (ABS) controls against three changes of 1 l of phosphate-buffered saline pH 7.4. The sera from MEP and GW₂ were taken directly from the patients then placed in sterile preservative-free containers. The titres of the sera were determined by indirect anti-immunoglobulin tests carried out on all the sera at the same time. The sera possessing anti-En^a antibodies were absorbed by incubating them with an equal volume of group O +ve cells for 1 h at 37 °C. Similar treatment of ABS did not alter the rates of invasion (data not shown). Successful absorption of these anti-En^a sera was confirmed by indirect antiglobulin testing using En(a+) cells.

* In these experiments the concentration of serum was increased from 50 to 100 µl per well.

sera on parasite multiplication was examined in several experiments (Table 3). The presence of antibodies on the red cell surface was confirmed by direct anti-immunoglobulin testing. There was a significant reduction in the number of parasites in cultures to which sera were added, regardless of the method of collection, either compared with ABS or, more importantly, with the same anti-En^a sera after absorption with En(a+) cells. The greater inhibition of invasion using the serum of GW correlated with its titre in an indirect antiglobulin test. The other sera used were of relatively low titre, having been taken several years after the individuals were sensitized.

To exclude the possibility that coating of red cells with anti-En^a antibody might reduce parasite invasion in a non-specific manner, various other sera directed against commonly occurring antigens present on red cells were examined in the same way. Two of these antibodies were directed against Rhesus antigens (anti-C and anti-c), one against the Kidd system (anti-Jk^b) and another against a Duffy determinant (anti-Fy^b). In no case was the number of parasites found in cells coated with antibody significantly less than in the controls (Table 4). On the other hand, there was a significant decrease in parasite numbers when sera from two patients who had recently

recovered from cerebral malaria were added to the cultures. Anti-immunoglobulin tests confirmed the presence of antibody on the surface of the cells incubated with antisera to red cell antigens, but not on the cells incubated with the patients' sera. Antibodies in the latter are thought to act via merozoite agglutination rather than by receptor blockade¹⁶.

Recent structural analysis of glyophorin A indicates that the M and N antigens are determined by its five amino-terminal amino acids and three oligosaccharides¹⁷. We further examined whether this most external portion of the glyophorin A molecule might be involved in the interaction with the parasite by comparing the relative rates of invasion of *P. falciparum* into cells bearing the M, the N and both antigens. The rates of invasion observed in MN, MM or NN cells were similar, indicating that the merozoite was unable to distinguish between these two antigens (Table 5).

These experiments have shown a significant reduction in invasion of En(a-) cells by *P. falciparum*. This may indicate either that there is a specific receptor site on the major sialoglycoprotein, or that there is a non-specific effect due to rearrangement of the membrane proteins in the absence of this major component. However, the presence of anti-En^a antibody caused

Table 4 Invasion of En(a+) cells by *P. falciparum* in the presence of antisera other than anti-En^a

Strain of parasite	ABS (control)	Parasites per 100 red cells					
		Anti-C	Anti-c	Anti-JK ^a	Anti-Fy ^a	JG	DH
A	8.2	7.7	8.4	8.2	7.7	4.2	0.0
A	3.8	3.6	4.1	4.0	3.7	0.0	0.0
B	5.7	6.5	7.0	6.5	6.6	NT	NT
C	9.1	7.9	7.8	5.5	8.8	4.5	0.3
C	5.6	2.0	5.3	4.1	5.3	4.8	2.3
Mean	Test Control	0.85 ± 0.13	1.05 ± 0.05	0.96 ± 0.08	0.99 ± 0.06	0.47 ± 0.18	0.11 ± 0.10

The methods used were as for Tables 1 and 3. Three strains of parasite were used: A, Uganda-Palo Alto; B, wild West African; and C, wild East African. The wild strains were obtained from infected patients returning to Oxford. Sterile, preservative-free antisera were obtained from the Oxfordshire Regional Blood Transfusion Service. Sera were also obtained, at least 1 month after completing chloroquine therapy, from two patients convalescing after severe *P. falciparum* infections (JG and DH). The relevant typing of the En(a+) cells used was as follows: ABO group O; Rh-positive (C⁺, D⁺, E⁺, c⁺, e⁺); M⁺, N⁺, Fy(a⁺), Fy(b⁺); Jk(a⁺), Jk(b⁻), Wr(a⁻), Wr(b⁺).

NT, not tested.

Table 5 Invasion by *P. falciparum* of cells expressing either the M or N or both antigens

MN cells (Control)	Parasites per 100 red cells MM cells (Test)	NN cells (Test)
8.8	9.1	9.8
7.5	6.6	7.8
6.4	6.7	6.5
Mean ($\frac{\text{Test}}{\text{Control}}$)	0.99 ± 0.05	1.06 ± 0.03

The methods were as for Table 1. The cells were obtained in CPD from the Oxfordshire Regional Blood Transfusion Service.

a significant reduction in parasite numbers in En(a+) cells which was prevented by previous absorption with En(a+) cells. This effect was not seen when other antibodies were used to coat the red cells. This finding suggests that the relationship between the parasite and the missing membrane component of the En(a-) cells is more specific. The fact that anti-En^a antibody (GW₂ and MEP) failed to reduce invasion of normal cells to the level found in En(a-) cells may have been due to low antibody titres resulting in insufficient blockade of this high density receptor (glycophorin A), of which there are at least 500,000 copies per cell¹⁸. Moreover the reduction in invasion caused by the low titre anti-En^a sera cannot exclude the role of the Wr^b antigen which is also recognized by this serum. Although these antisera could act by damaging the mature schizont-infected cell, by merozoite agglutination, or by premature lysis of infected En(a-) cells, these data, together with the reduced invasion of En(a-) cells, are more consistent with the anti-En^a sera acting via receptor blockade.

Of the many types of red cells that have been examined previously, only those containing haemoglobin S or predominantly haemoglobin C have shown rates of invasion inappropriate to their metabolic age¹⁹. En(a-) cells must now be added to this list. Miller has shown that trypsin-treated red cells are relatively resistant to invasion by *P. falciparum*⁴. Glycophorin A is one of the major targets for cleavage by this enzyme. These findings, together with the present studies, suggest that glycophorin could serve as a receptor for *P. falciparum*. The development of monoclonal antibodies against specific regions of these glycoproteins should allow this possibility to be explored further.

We thank Dr W. Wagstaff, Anna Pirkola, Dr R-M. Guévin, Mr V. Taliano and Mr M. Woodward for providing the numerous samples of cells, sera and reagents. We also thank Drs R. Sanger and P. Tippet for assistance. G.P. was supported by the Wellcome Trust and Rockefeller Foundation.

Note added in proof: Since submission of this letter, Perkins²³ has shown that glycophorin A in solution and Fab' fragments of antibody against glycophorin A will inhibit merozoite invasion.

Received 22 April; accepted 1 August 1981.

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Virus-induced diabetes mellitus: autoimmunity and polyendocrine disease prevented by immunosuppression

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Autoantibodies to cytoplasmic and surface antigens on pancreatic islet cells have been found in many newly diagnosed cases of insulin-dependent diabetes mellitus (IDDM)¹⁻⁹. Various autoantibodies (for example, against islet cells, adrenals, thyroid, gastric mucosa and intrinsic factor) have also been found in patients with polyendocrine disease^{1,2,8,9}. What triggers the production of these antibodies and their importance in the pathogenesis of these diseases are still unclear. Recently, we found in an animal model for studying polyendocrine disease¹⁰ that reovirus type 1 (reo-1) infected cells in both the pancreas and pituitary, and produced a disease characterized by hyperglycaemia and retarded growth¹⁰. Moreover, autoantibodies that reacted with antigens in pancreatic islets, the anterior pituitary and gastric mucosa were found, one of which reacted with insulin and another with growth hormone (GH). Using recombinant viruses¹¹ we showed that the S1 gene segment from reo-1 was required for the induction of autoantibodies to GH. We report here our search for additional autoantibodies, especially against surface antigens, and our efforts to evaluate the pathological contributions of the lytic effect of the virus on cells compared with the immunopathological response of the host. Our experiments showed that reo-1 induced autoantibodies that reacted with antigens on the surface of islet cells, thymocytes and a GH-producing cell line. In addition, we found that treatment with immunosuppressive agents depressed autoantibody production and prevented development of the polyendocrine disease.

Except where indicated, male and female SJL mice were infected intraperitoneally (i.p.) at 5-7 days of age with the Lang strain of reo-1 that had been passaged 10-14 times in pancreatic β-cell cultures¹⁰. Rabbit anti-mouse lymphocyte serum (ALS) and rabbit anti-mouse thymocyte serum (ATS) (MA Bioproducts, Walkersville, Maryland), with cytotoxic titres 1:6,400 and 1:1,600 respectively, and cyclophosphamide (Cy) (20 mg per kg)¹² were used as immunosuppressive agents. Single-point glucose tolerance tests (GTTs) were performed 60 min after i.p. injection of 2 mg glucose per body weight¹⁰. Plasma GH was measured by radioimmunoassay^{10,13}, and antibody to reo-1 was determined by a standard haemagglutination-inhibition technique¹⁴. Indirect immunofluorescence was carried out as described previously¹⁰.

Three different immunosuppressive agents were tested for their effect on the development of reovirus-induced diabetes. As shown in Fig. 1, reo-1 produced abnormal GTTs in SJL mice, but these abnormalities were reduced or prevented by treatment with ALS (Fig. 1a). Similarly, ATS and Cy prevented the development of reovirus-induced hyperglycaemia (Fig. 1b). Reo-1 also produced some glucose abnormalities in NFS mice and these abnormalities were suppressed by ALS (Fig. 1c). All eight experiments performed with reo-1 and immunosuppressive agents revealed similar trends, although in some experiments the reovirus-induced abnormal GTTs were much less marked than those observed in Fig. 1a and b.

Immunosuppression prevented not only the development of hyperglycaemia but also growth retardation. Table 1 shows that

virus-infected immunosuppressed mice gained weight at almost the same rate as uninfected controls, and that oily hair and steatorrhoea were greatly decreased by ALS. Mortality was also markedly reduced. Moreover, the infected mice with retarded growth had considerably lower plasma GH levels than the infected and immunosuppressed mice.

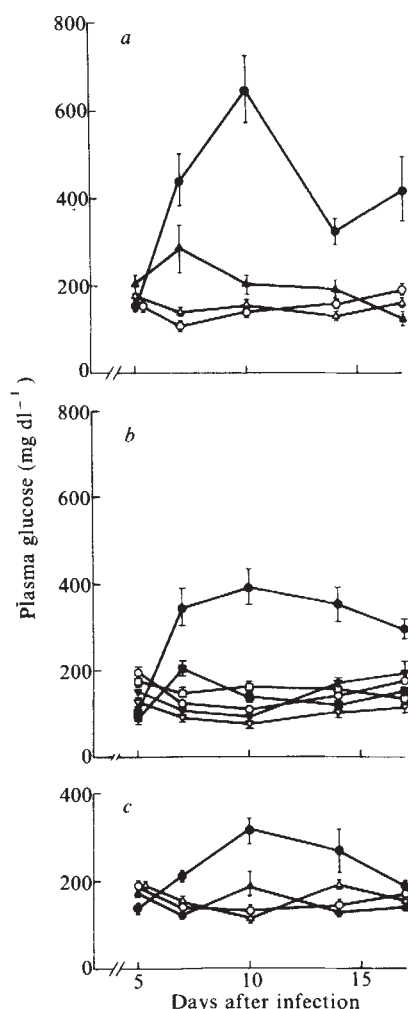


Fig. 1 Effect of immunosuppressive agents on glucose tolerance tests in reo-1-infected mice. Mice were infected with 5×10^5 plaque-forming units (PFU) of virus and single-point glucose tolerance tests were performed at different times thereafter. Immunosuppressive treatment consisted of three 0.1-ml i.p. injections of ALS or ATS on days 0, 4 and 7 after infection or a single i.p. dose of Cy 1 day after infection. 10 to 35 mice were tested at each time point in each group. The mean $\pm$ s.e.m. is represented by symbols and vertical lines. **a**, SJL mice: $\bullet$, reovirus-infected; $\circ$, uninfected; $\blacktriangle$, infected and treated with ALS; $\triangle$, uninfected and treated with ALS. **b**, SJL mice: $\bullet$, reovirus-infected; $\circ$, uninfected; $\blacksquare$, infected and treated with ATS; $\square$, uninfected and treated with ATS; $\blacktriangledown$, infected and treated with Cy; $\triangledown$, uninfected and treated with Cy. **c**, NFS mice: $\bullet$, reovirus-infected; $\circ$, uninfected; $\blacktriangle$, infected and treated with ALS; $\triangle$, uninfected and treated with ALS.

To see whether the immunosuppressive agents might be preventing clinical symptoms by inhibiting the autoimmune response, we measured the titre of autoantibodies in the sera of infected mice reacting with tissue sections from uninfected normal mice¹⁰. As shown in Table 2, autoantibodies to the anterior pituitary, islet cells and GH₃ cells (a rat cell line which produces both GH and prolactin)¹⁵ were greatly reduced, or not detected, in sera from reovirus-infected immunosuppressed mice. Immunosuppression also reduced the percentage of mice with autoantibodies to GH and insulin.

In addition to autoantibodies reacting with cytoplasmic antigens, autoantibodies to surface antigens on viable islet cells, GH₃ cells and thymocytes, but not mouse fibroblasts, were found by avidin-biotin immunofluorescence¹⁶. In our hands, this method is more sensitive than standard indirect immunofluorescence. Nonetheless, the titre of these antibodies was quite low and they reacted with only a small number (10–15%) of islet cells, GH₃ cells and thymocytes. In reovirus-infected immunosuppressed mice, these autoantibodies could not be detected. Immunosuppression also reduced, but did not eliminate, the antibody response to reo-1 (Table 2); this may account for the ability of the immunosuppressed animal to cope with the viral infection.

As previously reported, reovirus infection produced mild β -cell damage and insulinitis, with infiltration of mononuclear and plasma cells¹⁰. In the present experiments, immunosuppression with ALS greatly reduced the degree of insulinitis and β -cell damage (data not shown). Precisely how the viral infection triggers the autoimmune and inflammatory responses¹⁰ and what role, if any, autoantibodies to thymocytes have in this process are unclear.

The possibility that the immunosuppressive agents were preventing the polyendocrine diseases by inhibiting virus replication (such as by antiviral antibody in the immunosuppressive sera or induction of interferon), rather than by suppressing the autoimmune response, was evaluated by measuring virus growth *in vivo*. Figure 2 shows that the former possibility is unlikely as virus titres in homogenates of liver, heart and pancreas were approximately the same in untreated and ALS-treated mice at various times after infection.

Viral infections cause tissue damage by two principal mechanisms: they can directly lyse cells as a result of virus replication, and they can trigger in the host an immunopathological response^{17–19}. In the case of reovirus, both mechanisms may operate; infected cells are certainly destroyed as a result of virus replication and a recent study suggests that some cells may also be destroyed by cytolytic T lymphocytes specific for reovirus antigens on the surface of cells²⁰.

The present experiments suggest another possible mechanism by which reovirus might cause disease: the infection triggers the production of autoantibodies that react with antigens on the surface of normal uninfected cells or with cell products (for example hormones). These interactions could act pathologically by directly destroying hormone-producing cells or by binding to and reducing the concentration of hormones in the circulation.

In experimental animals, we previously showed that neither

Table 1 Reversal of reovirus-induced growth retardation, clinical symptoms and mortality by immunosuppression

Groups		Weight (g)		% Oily hair and steatorrhoea		% Mortality		GH (ng ml ⁻¹)
Infection	ALS	SJL	NFS	SJL	NFS	SJL	NFS	SJL
+	–	7.6 $\pm$ 2.3*	8.3 $\pm$ 2.5*	63	50	43	40	16 $\pm$ 8
+	+	12.3 $\pm$ 3.5†	13.7 $\pm$ 1.8†	0	0	25	13	48 $\pm$ 4
–	+	11.4 $\pm$ 1.0†	13.6 $\pm$ 1.0†	0	0	0	0	ND
–	–	14.1 $\pm$ 2.4	14.9 $\pm$ 2.2	0	0	0	0	41 $\pm$ 6

SJL and NFS mice were infected and treated with ALS as in Fig. 1. Each group consisted of 10–20 mice. Body weights given were for 17 days after infection (mean $\pm$ s.d.), the incidence of oily hair and steatorrhoea was determined 10 days after infection, cumulative mortality was assessed at 17 days after infection, and plasma GH 14 days after infection (mean $\pm$ s.e.m.). ND, not done.

* $P < 0.001$ compared with uninfected and untreated controls.

† Not statistically significant compared with uninfected and untreated controls.

Table 2 Effect of immunosuppression on autoantibody titres in reovirus-infected mice

Groups		Autoantibodies to								Antibody to reovirus
		Cytoplasmic antigens*			Surface antigens†			Hormones‡		
Infection	ALS	Anterior pituitary	Islets	GH ₃	Islets	GH ₃	Thymocytes	Insulin	GH	
+	-	32	8	16	4	2	8	56	89	256
+	+	2	2	<2	<2	<2	<2	16	20	16
-	+	<2	<2	<2	<2	<2	<2	0	0	<8
-	-	<2	<2	<2	<2	<2	<2	0	0	<8

Reo-1 infection and ALS treatment were as in Fig. 1. Autoantibodies were measured in pooled sera from 10 mice. Similar results were obtained with pooled sera from two other experiments. Titres for reovirus antibody are given as reciprocal antibody titres by haemagglutination-inhibition tests to reo-1 in pooled sera 17 days after infection.

* Bouin's-fixed sections of anterior pituitary and pancreas from uninfected mice, or acetone-fixed rat GH₃ cells were incubated with serial dilutions of mouse sera (obtained 14 days after infection) and stained with fluorescein-labelled anti-mouse IgG. Titres represent the reciprocal of the highest dilution of sera giving positive fluorescence¹⁰.

† Suspensions of cultured mouse SJL pancreatic islet cells, or rat GH₃ cells, or freshly prepared thymocytes from weanling SJL mice were incubated with pooled mouse sera obtained 14 days after infection and stained with biotin-conjugated anti-mouse immunoglobulin followed by fluorescein-labelled avidin¹⁶. Titres represent the reciprocal of the highest dilution of sera giving positive fluorescence.

‡ Individual sera from 10–20 mice per group (obtained 17 days after infection) were tested for antibodies to insulin and GH by an enzyme-linked immunosorbent assay¹⁰. Sera with titres >10 were considered positive and numbers represent per cent positive.

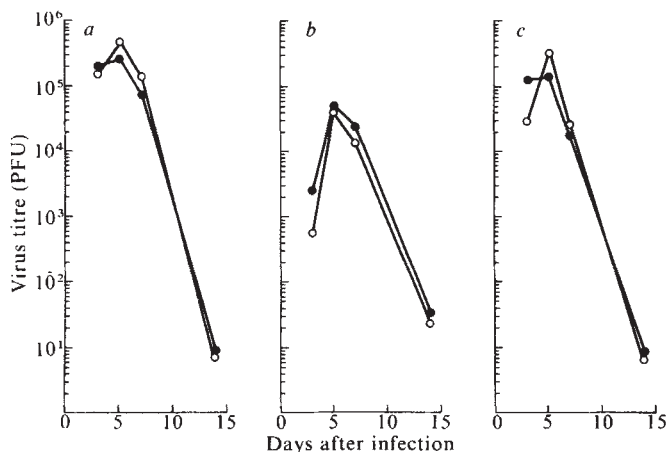


Fig. 2 Reovirus titre in ALS treated mice. SJL mice were infected with 5×10^5 PFU and treated with ALS as in Fig. 1. Virus titres in homogenates of liver (a), heart (b) and pancreas (c) were measured at different times after infection. Titrations were performed on L929 cells by a standard plaque assay¹⁰ and expressed as PFU per organ. Each point represents the mean titre of two to four individual mice. ○, Infected mice given ALS; ●, infected mice not ALS.

virus alone nor sub-diabetogenic doses of streptozotocin alone could produce hyperglycaemia, whereas treatment with both agents resulted in overt diabetes²¹. Our interpretation was that neither virus nor streptozotocin alone depleted the β -cell reserve sufficiently to result in diabetes. Reovirus-induced polyendocrine disease may also be the result of cumulative injury. Both the direct lytic effect of the virus and autoimmune pathology may be required to decrease the hormone reserve sufficiently to make the disease overt. The possibility that virus-induced autoimmunity contributes to the pathogenesis of other diseases merits investigation.

We thank Tecolia Brown and Royd Ellis for technical help and Edith Rian for editorial assistance.

Received 7 January; accepted 15 March 1982.

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Attenuated reovirus type 3 strains generated by selection of haemagglutinin antigenic variants

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Infection of suckling mice with mammalian reovirus type 3 results in fatal encephalitis associated with marked destruction of neurones^{1–4}. The viral tropism specific to neural cells is determined by the reovirus haemagglutinin^{3,4}. This haemagglutinin (HA), the σ 1 polypeptide, is encoded in the S1 dsRNA segment^{5,6}. In addition to determining cell and tissue tropism, the viral haemagglutinin determines humoral and cellular immune specificity^{7–10}, binding to cellular microtubules¹¹ and inhibition of cellular DNA synthesis¹². To elucidate the structural basis for the functions of the reovirus type 3 HA, we have recently isolated and characterized several anti-HA monoclonal antibodies, which we have used to show that there are distinct functional domains on the reovirus type 3 HA¹³. One group of monoclonal antibodies neutralizes viruses while a second inhibits haemagglutination. Thus there are distinct HA functional domains involving haemagglutination and neutralization. One of the neutralizing monoclonal antibodies was used to select reovirus antigenic variants that were no longer neutralized by the monoclonal antibodies. These 'variant' viruses were tested for their neurovirulence by determining their capacity to replicate in mouse brains, leading to fatal encephalitis. We report here that all variants are markedly less virulent than the parental type 3 virus from which they were selected. These findings demonstrate directly the critical role of the viral haemagglutinin in neurovirulence and show that variant viruses that are altered at a site recognized by neutralizing antibody cannot grow efficiently in neural tissue.

Reovirus serotype 3 viruses with antigenically altered HA proteins were selected by incubating the neurovirulent virus stock (Dearing strain) with excess neutralizing monoclonal antibody (designated G5). Viral plaques that appeared on the plates were isolated and passaged a second time in the presence of G5 antibody to eliminate clones that were not resistant to the antibody. Resistant viruses (variants) were then grown in mouse L cells to give virus stocks. Three variant viruses (A, F and K) isolated in this manner were tested for their relative ability to resist neutralization by the G5 monoclonal antibody (Table 1). As reported previously, the parental reovirus type 3 (Dearing) strain was neutralized efficiently by G5 (ref. 13). All three variants showed an altered response to G5 antibody: the F and K variants were totally resistant to the antibody while the A variant was neutralized to a level intermediate between that of the Dearing parent and the F and K variants. For example, the A variant was neutralized at 1:20 and 1:100 dilutions of G5, but the plaque reduction at 1:500 was of borderline significance. The Dearing strain was neutralized at all the dilutions tested. Previous studies have shown that the G5 antibody neutralizes the Dearing strain at dilutions up to 1:12,500 (ref. 13). In contrast to the results with the G5 monoclonal antibody, the three variants were indistinguishable from the parental reovirus type 3 Dearing virus in neutralization tests using a type-specific hyperimmune antiserum (Table 1). These data indicate that all three variants encode an haemagglutinin having antigenic alterations that are readily detected with monoclonal antibodies but not with a standard hyperimmune antiserum.

To determine if the variants were altered in terms of virulence, we compared them with regard to their relative ability to cause fatal neurological disease in suckling mice. The type 3 Dearing parent is highly neurovirulent; as few as 10 plaque-forming units (PFU) inoculated intracerebrally (i.c.) in suckling mice kill 50% of mice by 14 days while higher doses kill 100% (ref. 3; Table 2). To compare the neurovirulence of type 3 Dearing with the variant viruses, we injected varying doses of virus into suckling mice and determined the survival pattern for each infected group of animals (Table 2). The F and K

Table 1 Neutralization of reovirus serotype 3 (Dearing) and HA antigenic variants by monoclonal antibody and hyperimmune antiserum

Antibody	Dilution of antibody	Dearing strain	% Inoculum resistant to antibody Variant strains		
			A	F	K
G5	1:20	0	21	100	88
	1:100	0	18	100	100
	1:500	5	48	100	100
Hyperimmune	1:200	6	7	4	3
	1:400	5	4	2	4
	1:800	14	4	5	9

Neutralization tests were performed as described previously¹³. Briefly, dilutions of antibody were incubated with 100 PFU of virus at 34 °C for 1 h. These mixtures were then inoculated on to mouse L cells. The G5 antibody was used to select the variants and was used in these experiments as a purified IgG fraction¹³. Values represent the percentage of plaques observed in the presence of antibody relative to a phosphate-buffered saline control. The hyperimmune antiserum is a reference goose anti-reovirus type 3 antiserum obtained from the National Institute of Allergy and Infectious Diseases (V703-501-570).

variants had LD₅₀s (lethal dose for 50% of the population) values of 3×10^7 and $>3 \times 10^7$ PFU, respectively, while the A variant had an intermediate LD₅₀ of 1.8×10^5 PFU. These results indicate that all the variants are at least 10,000 times less neurovirulent than the Dearing strain from which they were derived. Interestingly, the A variant that was intermediate in antigenic reactivity, also exhibited an intermediate LD₅₀ (1.8×10^5) whereas the F and K variants, which were not neutralized by G5, showed the greatest reduction in virulence. These data suggest that the loss of antigenic reactivity with the G5 antibody is directly related to the neurovirulence.

Because neurovirulence is correlated with the ability of reovirus type 3 to replicate in suckling mouse brains, we analysed the growth of type 3 reovirus and the three variants in mouse brains (Fig. 1). The reovirus type 3 (Dearing) virus grows to high titres in the brain before death of the infected mice^{3,4}. The virus yield in these experiments was $\sim 1 \times 10^9$ PFU ml⁻¹, but titres were often $\geq 1 \times 10^{10}$ PFU ml⁻¹. The three variants differed significantly from the Dearing strain in their ability to grow in mouse brains during the acute infection, particularly after the fifth day post-inoculation (Fig. 1). The F and K variants showed most reduction in growth while the A variant was again intermediate between the F and K variants and the Dearing virus.

To determine whether the inability of the three variants to grow in brains was due to a general defect of growth or was

Table 2 Neurovirulence of reovirus serotype 3 (Dearing) and HA antigenic variants A, F and K

Inoculum (PFU)	Dearing strain	% Mice alive on day 14 post-infection Variant strains		
		A	F	K
1×10^1	50	100	100	100
1×10^2	0	100	100	100
1×10^3	0	100	100	100
1×10^4	NT	100	100	100
1×10^5	NT	90	100	100
3×10^5	NT	0	100	100
3×10^6	NT	0	90	100
3×10^7	NT	0	50	90
LD ₅₀	10^1	1.8×10^5	3×10^7	$>3 \times 10^7$

Groups of CD mice (24–48 h old) were injected i.c. with varying doses of virus and observed for 14 days^{3,4}. Animals began to die between days 8 and 10 in most cases. Values represent the percentage of mice alive at day 14 post-infection. NT, not tested. The LD₅₀ is the dose of virus required to kill 50% of the animals in each group by day 14. LD₅₀ values were calculated by the Reed and Muench method¹⁵.

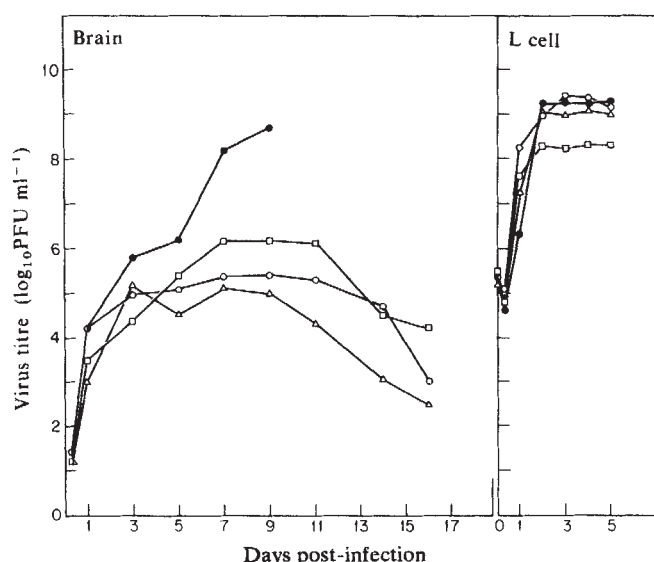


Fig. 1 Growth patterns of reovirus type 3 (Dearing, ●) and antigenic variants A (□), F (○) and K (Δ) in mouse brains (left) and mouse L cells in tissue culture (right). Virus growth in mouse brains was determined by injecting all mice i.c. with 1×10^3 PFU of virus. Three mice were killed at each time point and the virus titres in the individual brains were determined by titration of brain homogenates on mouse L cells at 37 °C as described previously⁴. Each value represents the average of three titrations. Virus growth in mouse L cells was determined by infecting monolayers of 2×10^5 cells at a multiplicity of infection of 5 PFU per cell. Triplicate samples were taken for titration at each indicated time point¹⁶.

specific to growth in the brain, we examined the replication cycle of the variants and Dearing virus in mouse L cells (Fig. 1). Both reovirus type 3 Dearing and the three variants grew to a high titre. The variants were also not temperature-sensitive for growth in L cells (data not shown). Examination of brains infected with variants A, F and K showed a marked localization of tissue destruction in parts of the hippocampus. In contrast, infection with the Dearing strain caused tissue destruction not only in the hippocampus but also diffusely in the cortex and brain stem (unpublished data). Thus the pattern of restricted growth in the brain was not due simply to a general defect in the ability of the three variants to replicate, but to growth in restricted sites in the brain.

Thus, antigenic variants of reovirus type 3 selected by monoclonal antibodies directed against the major neutralization site, have markedly different levels of neurovirulence and capacity to grow *in vivo*. These experiments directly indicate that the reovirus type 3 haemagglutinin is a major determinant of neurovirulence, and imply that particular regions of the HA have critical roles in virulence. The site chosen for selection of

the variants, the major neutralization determinant on the HA molecule, is probably the polypeptide region involved in binding to neurone receptors¹⁴. Other studies have shown that this region is also the site on HA that is recognized by cytotoxic T lymphocytes (unpublished data), and is involved in binding to cell receptors¹⁴. Furthermore, we have recently demonstrated that these variants induce cytolytic T lymphocytes as well as neutralizing antibody (unpublished data). Further data on the nature of the alterations in the haemagglutinin polypeptide of these variants should enable us to define the role of haemagglutinin in viral virulence more precisely. In addition, the use of monoclonal antibodies to generate attenuated viral strains may serve as a rapid and simple step for developing viral vaccines.

We thank Elaine Freimont for technical assistance and David Knipe and Robert Finberg for their helpful suggestions concerning the manuscript. This work was supported by grant 2 ROI AI 13178-06 from the National Institute of Allergy and Infectious Diseases, NIH. D.R.S. was supported by a fellowship from the Juvenile Diabetes Foundation.

Received 28 October 1981; accepted 19 March 1982.

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Non-neutralizing monoclonal antibodies can prevent lethal alphavirus encephalitis

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Among the heterogeneous population of antibodies specifically induced during many acute viral infections, those having virus-neutralizing activity *in vitro* are generally considered to be most important for recovery and immunity to reinfection. Similarly, the ability to stimulate production of circulating neutralizing (NT) antibodies is a major criterion for evaluating the immunoprophylactic potential of many antiviral vaccines. Although there is obviously an association between NT antibody induction and host resistance, we present here data which indicate that other virus-specific antibodies lacking NT function may be equally important in conferring protective immunity to alphaviruses. We used monoclonal antibodies against Sindbis virus (SV) to demonstrate that passive protection of SV-infected mice from fatal paralytic central nervous system (CNS) disease may be mediated not only by antibodies which neutralize the infectivity of extracellular virus particles but also by those lacking this capacity, which react preferentially with virus-infected cells.

Sindbis virus, the prototype of the alphaviruses, contains two distinct envelope glycoproteins, E1 and E2, which exist as a complex, and a nucleocapsid protein, NC. It has been shown that antibodies raised in rabbits against each of the individually isolated and purified glycoproteins of SV possess distinctly separable activities *in vitro*². Antibodies to E1 (the viral haemagglutinin) inhibit haemagglutination by SV, whereas only antibodies to E2 significantly neutralize viral infectivity. Antibodies specific for the internal NC do not react with intact

virions. Both haemagglutination-inhibiting (HI) and NT antibodies are induced during productive infections with SV as well as with other alphaviruses³, but their respective roles in mediating host recovery are not clear.

Recently⁴, we described the preparation of a large panel of monoclonal antibodies against the parental AR339 strain of SV (SV_p), some of which were used to define serological and biochemical relationships between SV and certain other alphaviruses. In the present study, six anti-E1, six anti-E2, and one anti-NC monoclonal antibodies were selected for further characterization and each was assessed for its protective capacity *in vivo*. Because, by any route of inoculation, SV_p produces an abortive immunizing infection in adult mice, a neuroadapted (na) variant⁵ of SV_p was used to intracerebrally (i.c.) challenge C57BL/6J (B6) mice previously given monoclonal antibodies. When given i.c. to non-immune adult B6 mice, SV_{na} produces paralysis and fatal encephalitis.

To prepare monoclonal antibodies, spleen cells from BALB/cJ mice immunized with infectious SV_p were fused with P3X63-Ag8 myeloma cells, and hybrid cells were cloned by limiting dilution in hypoxanthine-aminopterin-thymidine medium⁶. Clones producing antibodies to SV_p were selected by solid-phase radioimmunoassay⁷ of supernatants. Positive cultures were subcloned in soft agar and tested again by radioimmunoassay for anti-SV activity. Individual hybrid clones were expanded *in vitro*, and then inoculated into groups of pristane-primed BALB/cJ mice⁸. The resulting ascitic fluids derived from each hybrid clone were pooled, frozen in aliquots, and used in subsequent experiments. The specificity of each monoclonal antibody was determined by radioimmunoassay reactivity with individual SV_p structural proteins which had been separated by isoelectric focusing as previously described². Specificity was confirmed by immunoprecipitation of radio-labelled virus, followed by polyacrylamide gel electrophoresis.

The isotypes of the monoclonal antibodies were determined by an indirect immunofluorescence procedure in which glass slides bearing acetone-fixed, SV_p-infected BHK-21 cells were exposed sequentially to predetermined concentrations of monoclonal antibody, rabbit antiserum (Litton Bionetics) specific for individual mouse immunoglobulin heavy-chain isotypes (IgM, IgG1, IgG2a, IgG2b or IgG3), and fluorescein-conjugated goat

Table 1 Properties of anti-SV_p monoclonal antibodies

No.	Monoclonal antibody		IF	Reactivity with SV _{na}	
	Specificity	Isotype		NT titre	% C'-dependent cytolysis
Protective <i>in vivo</i>					
12	E2	IgG2b	+	3,200	55.3
43	E2	IgG2a	+	800	21.1
49	E2	IgG2a	+	1,000	47.7
1	E1	IgG2a	+	<10	29.4
2	E1	IgG2a	+	<10	16.8
7	E1	IgG2a	+	<10	29.1
38	E1	IgG2a	+	<10	32.6
31	E1	IgG2b	+	<10	28.5
Non-protective <i>in vivo</i>					
23	E2	IgG3	+	<10	2.1
8	E2	IgG2a	—	<10	ND
10	E2	IgG2a	—	<10	ND
13	E1	IgG1	+	<10	-1.3
3	NC	IgG3	+	<10	0.3

Specific binding by monoclonal antibody to SV_{na}-infected BHK-21 cells was determined by immunofluorescence (IF). Acetone-fixed cells on glass slides were overlaid with monoclonal antibody for 30 min, washed and then overlaid with fluorescein-labelled goat anti-mouse immunoglobulins (Cappel) for 30 min, then washed again. Each reagent was used at a 1:20 dilution in buffered saline, pH 7.4. All positive monoclonal antibodies exhibited bright perinuclear and cytoplasmic IF; antibodies 8 and 10 were completely negative. No IF was observed for uninfected control cells. Using the same procedure, all monoclonal antibodies including 8 and 10, were positive on SV_{na}-infected cells. To demonstrate C'-dependent antibody-mediated cytolysis, BHK-21 cells were infected (or mock-infected) in suspension with virus at a multiplicity of 6 PFU per cell, labelled with ⁵¹Cr, washed and seeded into flat-bottomed 96-well tissue culture plates (Costar). Each well received an aliquot (100 µl) containing 2 × 10⁴ cells in RPMI-1640 supplemented with 10% fetal bovine serum. To triplicate wells were added monoclonal antibody (previously heated at 56°C for 30 min) and guinea pig C' at final concentrations (v/v) of 2.5% and 5.0%, respectively, in a total volume of 200 µl per well. After 6 h incubation at 37°C in air containing 5% CO₂ (9 h post-infection) supernatants from each well (100 µl) were counted individually for radioactivity, and the mean % virus-specific cytolysis was calculated as described previously¹⁵; s.e.m. never exceeded 3%. Monoclonal antibody-mediated release of label from cells in control wells (uninfected cells + C', and infected cells + heat-inactivated C') did not exceed 2.8%. In medium alone, 17.9% and 16.7% of total incorporated label were released by infected and uninfected cells, respectively.

anti-rabbit immunoglobulins (Cappel). Slides were washed exhaustively after the application of each reagent.

Each monoclonal antibody was tested, in the presence of 5% (v/v) guinea pig complement (C'), for its ability to neutralize SV infectivity and to mediate C'-dependent lysis of SV-infected BHK-21 cells. We measured neutralizing activity against 100 plaque-forming units (PFU) of each virus preparation (SV_p and SV_{na}), using a conventional 'constant virus-varying antibody' assay⁸. The reciprocal of the highest dilution of a monoclonal antibody which produced a ≥80% reduction in PFU was designated its NT titre. SV-specific antibody-mediated cytolysis was determined in a 6-h ⁵¹Cr-release assay (Table 1).

To initially screen monoclonal antibodies for their protective capacity, each was given intraperitoneally (0.1 ml per mouse) to five female B6 mice, 6–8 weeks old, 24 h before challenge i.c. with 1,000 PFU of SV_{na}. All, or most mice in eight such groups resisted challenge, whereas all of a control group of infected non-immune mice, died. As shown in Table 1 (upper panel), passive protection by three anti-E2 monoclonal antibodies correlated with their having measurable NT activity against SV_{na}, although all such antibodies were capable of neutralizing SV_p (data not shown). In contrast, although none of the six anti-E1 monoclonal antibodies had NT activity against either SV_p or SV_{na}, five were protective. Sindbis virions acquire their envelopes by budding from host-cell plasma membranes which express both E1 and E2 during viral morphogenesis^{9,10}. Thus, irrespective of their glycoprotein specificity, all protective monoclonal antibodies shared an ability to bind SV_{na}-infected cells as shown by immunofluorescence and, as might be expected from their IgG2a and IgG2b isotypes, by C'-dependent cytolysis (Table 1).

The results of these initial passive protection tests were confirmed using larger numbers of B6 mice; protective mono-

clonal antibodies either prevented or delayed both paralytic disease and death (Table 2, upper panel). Measurements of infectious SV_{na} in individual brains obtained at intervals after challenge revealed that CNS infections were generally attenuated, rather than prevented, in mice which had received protective monoclonal antibody. On day 3 of infection, when brain virus titres were maximal in mice from groups given either non-protective or no monoclonal antibody, brains of mice from the protected groups contained at least 100-fold less virus (Table 2, lower panel). Furthermore, by day 7 of infection, when all unprotected mice were paralysed and/or moribund, infectious virus was no longer detectable in brains of protected mice (data not shown).

All monoclonal antibodies, whether protective or not, had similar titres (>10⁴) against SV_p in solid-phase binding assays (data not shown), and the failure of a monoclonal antibody to protect *in vivo* could not be attributed, in any case, to transfer of insufficient antibody. Certain monoclonal antibodies may be nonprotective, however, due to antigenic differences between SV_p and SV_{na} that result in undetectable or low-affinity binding to SV_{na}. Of the three anti-E2 monoclonal antibodies which failed to protect the mice, none neutralized SV_{na} and only one (no. 23) was reactive with SV_{na}-infected cells by immunofluorescence (Table 1, lower panel). The non-C'-fixing isotype (IgG3) of the latter would explain its failure to mediate SV-specific cytolysis *in vitro* but the fact that all three neutralized SV_p infectivity, even in the absence of complement (data not shown), may mean that passive protection conferred by anti-E2 antibodies depended more on their having NT activity against SV_{na} and less on their isotypes. On the other hand, it seems significant that the isotype (IgG1) of the single non-protective anti-E1 monoclonal antibody (no. 13) is one which does not mediate immunospecific cytolysis by the classical complement pathway¹⁰. If, in fact, protection by anti-E1 antibodies, which do not neutralize SV, is effected solely through this C'-dependent cytolytic mechanism, it would be restricted to only those isotypes capable of fixing complement. The importance of the latter in antibody-mediated protection against SV is also suggested by the impaired virus clearance previously noted in mice which are deficient in either the C3 (ref. 11) or C5 (ref. 12) complement components.

The present data suggest two specific but functionally different humoral mechanisms of SV elimination *in vivo*: (1) neutralization of infectivity by antibodies which bind to E2 determinants on extracellular virions, a process which can occur *in vitro* in the absence of C'; and (2) C'-dependent lysis of

Table 2 Prevention of paralysis and death of SV_{na}-infected mice by anti-SV_p monoclonal antibodies

No.	Monoclonal antibody specificity	Paralysis		Mortality		log ₁₀ PFU per g brain on day 3
		P/T	Mean day of onset	D/T	Mean day of death	
12	E2	0/10	—	0/10	—	ND
43	E2	0/15	—	0/15	—	ND
49	E2	3/20	14	1/20	12	<2.0, 4.3, 2.7
1	E1	3/15	11	1/15	18	ND
2	E1	0/14	—	0/14	—	5.2, 4.6, 3.2
7	E1	0/14	—	0/14	—	5.2, 6.5, 5.5
38	E1	0/25	—	0/25	—	<2.0, <2.0, 2.8
31	E1	7/13	7	2/13	9	ND
23	E2	10/10	5	9/10	9	ND
8	E2	15/15	5	14/15	8	7.4, 7.7
10	E2	10/10	5	10/10	9	ND
13	E1	14/14	6	13/14	8	ND
3	NC	15/15	5	15/15	10	7.4, 7.5
None	—	19/20	6	18/20	9	7.7, 7.6

Groups of 6–8-week-old female C57BL/6J mice were given 0.1 ml monoclonal antibody (in 0.5 ml saline) intraperitoneally, challenged i.c. 24 h later with 1,000 PFU of SV_{na} and then observed for at least 21 days. Results are expressed as the proportion of mice that developed hind limb paralysis (paralysed/total, P/T) and died (dead/total, D/T). Brains of mice in selected groups were frozen 1, 3, 5 and 7 days after infection and assayed individually for SV PFU on BHK-21 cells; results are shown for day 3, when maximum brain titres occurred in non-immune control mice.

SV-infected cells mediated by either neutralizing or non-neutralizing antibodies (of restricted isotypes) which bind, respectively, to E2 and E1 determinants on the cell surfaces. It is likely that one or both of these mechanisms involve(s) the recognition of more than one viral glycoprotein determinant. For example, all six anti-E1 monoclonal antibody were capable of binding to SV-infected cells (Table 1) but only two (1 and 31) had significant HI activity (data not shown).

The ability of five anti-E1 monoclonal antibodies to effect cytolysis, but not neutralization, in the presence of C' suggests that one or more E1 determinants expressed on the plasma membrane of infected cells are not similarly expressed on infectious SV particles. That non-NT anti-E1 antibodies did not recognize infectious SV was indicated further by their failure to neutralize virus in the presence of polyvalent anti-mouse-immunoglobulin antiserum which, in parallel assays, augmented the activity of NT antibodies (data not shown).

During viral morphogenesis, glycoproteins of SV and other alphaviruses undergo configurational changes associated with the cleavage of PE2 (the precursor of E2) and the subsequent formation of the virion E1/E2 complex^{9,13}. These changes may cause certain E1 and/or E2 determinants expressed initially on infected cells to become modified or cryptic on mature virions. Therefore, it would follow that the determinant specificities of antibodies induced by a given alphavirus glycoprotein would depend, in part, on the protein being either cell- or virion-associated when presented to the immune system. In this context, our finding that the protective non-neutralizing anti-E1 monoclonal antibody was functionally specific for SV-infected cells suggests that the induction of such antibodies might be enhanced by immunization with infectious rather than inactivated virus.

Our results also bear on the long-standing phenomenon of cross-protective immunity between heterologous alphaviruses in the absence of antibodies having corresponding cross-reactive NT activity (for review see ref. 3). Although the basis of this phenomenon has been ascribed to cross-reactive virus-specific effector T cells¹⁴, the participation of a humoral mechanism is suggested by the fact that monoclonal antibodies to the E1, but not the E2 glycoprotein, of SV exhibit serological cross-reactivity with other alphaviruses⁴. Whether these same non-neutralizing, monoclonal antibodies also mediate heterologous protection *in vivo* is a subject for future investigation.

We thank M. K. Gentry for preparing anti-SV monoclonal antibodies, Celia Jarvis for help in preparing the manuscript, and Dr J. F. Smith for his critical comments. This work was supported by the US Army Research and Development Command and USPHS grant NS 17741.

Note added in proof: We have recently found that four anti-SV monoclonal antibodies (including numbers 31 and 38 above), which bind three distinct determinants on E1, cross-react with western equine encephalitis virus and protect mice from lethal intraperitoneal challenge with this alphavirus.

Received 23 November 1981; accepted 5 March 1982.

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Specific opiate receptors on isolated mammalian gastric smooth muscle cells

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The predominant opioid peptides of the gut, Met-enkephalin and Leu-enkephalin are confined to neurones of the myenteric plexus^{1–4}. Axonal projections from these neurones innervate the circular muscle layer running parallel to the smooth muscle cells⁵. An opioid tridecapeptide (dynorphin), first isolated from the porcine pituitary⁶, is found in neurones of the submucosal plexus in the guinea pig; axonal projections from these neurones also innervate the circular muscle layer^{7,8}. Dynorphin consists of an N-terminal Leu-enkephalin sequence coupled to a C-terminal octapeptide by an Arg-Arg sequence. Neural receptors for all three opioid peptides and for opiate narcotics have been identified in the myenteric plexus where they cause hyperpolarization of neurones^{9,10}, inhibition of acetylcholine (ACh) release¹¹ and blockade of muscle contraction induced by field stimulation^{12–16}. Measurements of motor activity in circular muscle where enkephalinergic fibres are most abundant, suggest that opiates also have a direct contractile action^{17–20}. We have tested this possibility in isolated gastric smooth muscle cells devoid of neural elements^{21,22}, and report here the presence of specific high-affinity opiate receptors on such cells of the guinea pig. The receptors mediate contraction as measured by image-splitting micrometry, and exhibit a rank order of potency and absolute values similar to those reported for neural receptors of the myenteric plexus. In view of the distribution of enkephalinergic fibres predominantly to gut smooth muscle, our results strongly support a direct neuro-transmitter role for enkephalins in this region.

Smooth muscle cells were isolated from the stomach of the guinea pig as described in detail elsewhere²³ (see Fig. 1 legend). The morphometric profile of freshly dispersed cells is illustrated in Fig. 1 (mean cell length $\pm$ s.e.m. = 109.3 ± 1.2 μ m; $n = 52$). Exposure of the cells to maximally effective concentrations of Met-enkephalin, Leu-enkephalin or morphine for 30 s shifted the curve to the left, causing the appearance of a greater proportion of shorter, contracted cells. The contractile response to a given dose of agonist was determined from the fractional (percentage) or absolute decrease in mean cell length compared with controls.

The kinetics of contractile response to Met-enkephalin and other opiates were similar to those reported for other contractile agents such as ACh, COOH-terminal octapeptide of cholecystokinin (CCK-OP) and gastrin-17²². The response was immediate, reached a peak in 30 s and reverted to a sustained plateau for at least 8 min (Fig. 2). Figure 3 shows dose-response curves constructed for opioid peptides and morphine using the values

Table 1 Maximum contractile responses to opiate agonists

	Maximal contractile response (% decrease in cell length)	Potency [D_{50} (M)]
Dynorphin	33.7 ± 1.9 ($n = 7$)	10^{-10}
Met-enkephalin	34.1 ± 1.4 (9)	1.2×10^{-9}
D-al ² -met-enkephalinamide	30.1 ± 0.9 (5)	6.0×10^{-9}
Morphine	29.7 ± 1.3 (5)	2.0×10^{-8}
Leu-enkephalin	30.0 ± 3.6 (7)	1.2×10^{-7}

of the 30-s peak responses. At the highest doses used, all opiate agonists elicited maximal contractile responses similar to those previously reported for 10^{-9} M CCK-OP and 10^{-7} M ACh^{21,22} (30–34% decrease in mean cell length) (Table 1). The order of potency as determined from the dose required to produce one-half of the maximal response (D_{50}) was dynorphin > Met-enkephalin > D-Ala²-Met-enkephalinamide > morphine > Leu-enkephalin. Note that the widest difference in potency (1,200-fold) was between Leu-enkephalin and dynorphin, which includes the sequence of Leu-enkephalin in its NH₂-terminal; this raises the possibility that tryptic cleavage of dynorphin to Leu-enkephalin terminates the action of dynorphin as a putative neurotransmitter. The higher potency of Met-enkephalin relative to its synthetic analogue suggests minimal or no degradation of Met-enkephalin in muscle cell suspensions²³. If this assumption is correct, the potency as determined in isolated muscle cells should reflect the true

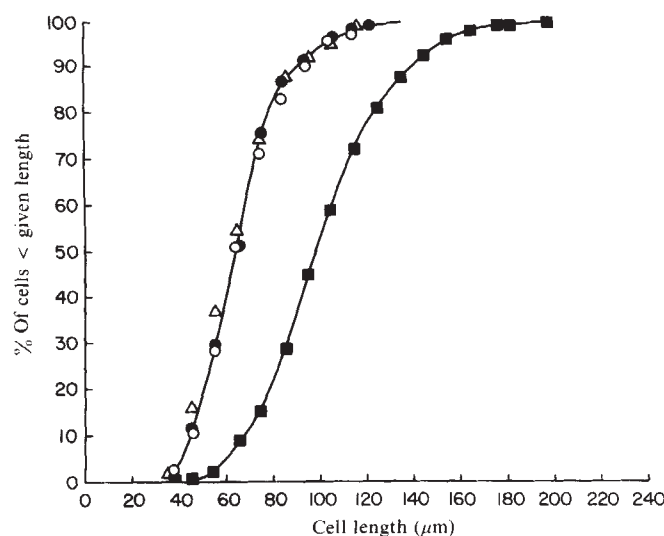


Fig. 1 Distribution of smooth muscle cell lengths in the control state (■) and after 30 s exposure to Met-enkephalin (●), Leu-enkephalin (△) or morphine (○). The shift of the curve to the left indicates the appearance of a greater proportion of shorter, more contracted cells. Cells were obtained from the circular muscle layer of the stomach of guinea pigs by two sequential 45 min incubations of muscle strips in Krebs bicarbonate buffer containing 0.1% collagenase (Worthington CLS, Type II) and 0.01% soybean trypsin inhibitor. The partly digested strips were washed with 50 ml of enzyme-free buffer and re-incubated for 30 min to allow cells to disperse spontaneously. The cells were collected by filtration through 500 μ m Nitex then examined within 30 min of dispersion. Aliquots (2.5×10^4 cells in 0.5 ml) were incubated for 30 s with 0.1 ml of a solution containing agonist, and the cells were then fixed rapidly with acrolein. Incubation periods in the range 15 s to 8 min were used to examine the kinetics of contraction. Control cells were treated in the same manner but without added agonist. In the control state and for each test modality, the lengths of 50 cells in sequential microscopic fields were measured by image-splitting micrometry. Contraction was determined from the percentage decrease in mean cell length compared with control.

structure-activity status of Met-enkephalin and other opioid peptides.

Naloxone (3×10^{-10} M) caused a parallel rightward shift of the dose-response curve for Met-enkephalin without affecting maximal response (Fig. 4). A higher dose of naloxone (3×10^{-9} M) caused a further shift of the dose-response curve and 10^{-7} M abolished the response to all doses of Met-enkephalin. We compared the effect of 3×10^{-10} M naloxone against the highest effective doses of opiate agonists (10^{-6} M for dynorphin and Met-enkephalin; 10^{-5} M for morphine and Leu-enkephalin). Naloxone was a more effective inhibitor of the responses to morphine and Leu-enkephalin (83–85% inhibition) than of the responses to dynorphin and Met-enkephalin (34 and 24% inhibition, respectively). Naloxone inhibited

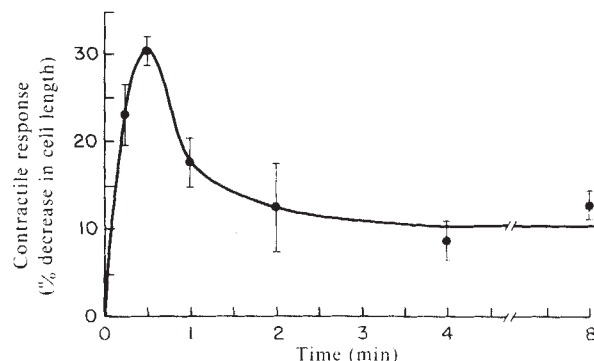


Fig. 2 Kinetics of contraction in response to 10^{-5} M Met-enkephalin. The prompt rise to a peak in 30 s and reversal to a sustained contractile plateau was similar to that previously found for non-opiate agonists^{21,22}. Each point is the mean $\pm$ s.e.m. of four experiments.

specifically the response to opiate agonists; it affected neither the response to CCK-OP nor the response to ACh. Conversely, dibutyl cyclic GMP (10^{-3} M), a specific antagonist of the CCK-gastrin receptor in neural, muscular and epithelial tissues^{24,25}, inhibited the contractile response to CCK-OP but had no effect on the response to the two opiate agonists tested (Met-enkephalin and morphine) or on the response to ACh²². Atropine (5×10^{-10} – 5×10^{-8} M) also had no effect on the response to 10^{-6} M Met-enkephalin or morphine, although it abolished the response to ACh²².

There was a remarkable similarity not only in the rank order but also in the absolute potencies of opiate agonists acting on isolated smooth muscle cells and nerve fibres of the guinea pig myenteric plexus^{8,13,26,27}. In both tissues, dynorphin exhibited the highest potency ($D_{50} \sim 10^{-10}$ M) and Leu-enkephalin and morphine, the lowest potency ($D_{50} \sim 10^{-7}$ M). The potency of Met-enkephalin in isolated muscle cells was much closer to that of dynorphin than to that of Leu-enkephalin. The high potencies of dynorphin and Met-enkephalin and the relative resistance

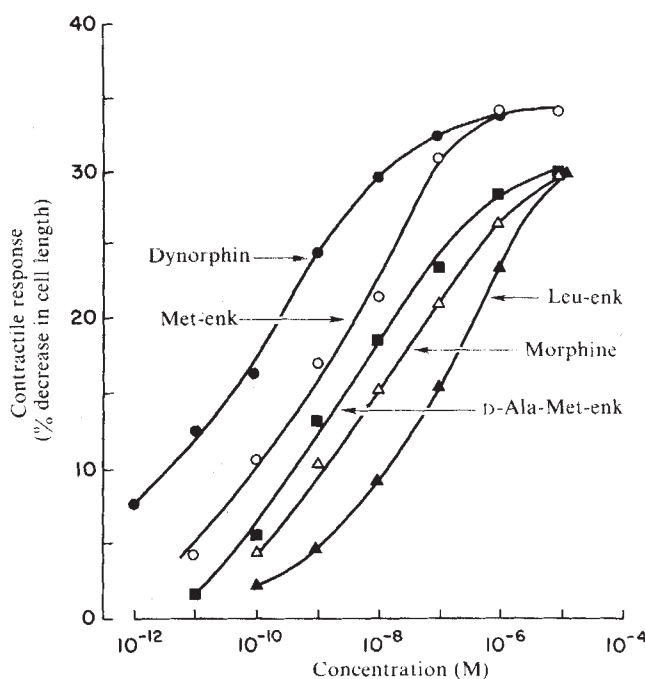


Fig. 3 Dose-response curves for the contractile effects of opioid peptides and morphine on isolated smooth muscle cells. Maximal responses and D_{50} s are given in Table 1. Each point is the mean of 4–9 experiments.

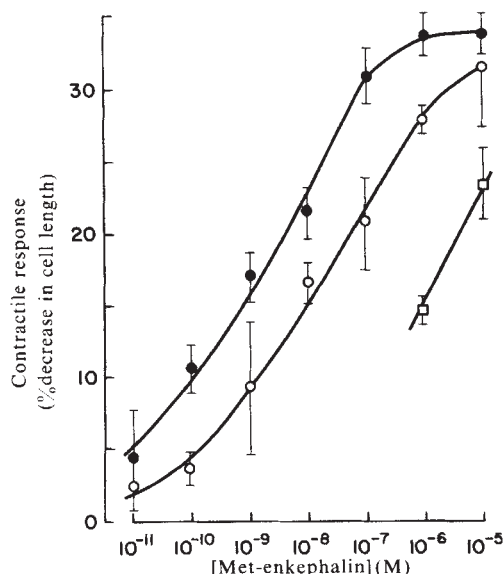


Fig. 4 Dose-response curves for the contractile effects of Met-enkephalin alone (●) or in combination with naloxone (○, 3×10^{-10} M; □, 10^{-9} M naloxone). Each point is the mean $\pm$ s.e.m. of four experiments.

of their effects to naloxone in isolated muscle cells suggest that the receptor(s) with which they interact are different from those for morphine (μ receptors). Chavkin and Goldstein²⁶ applied selective protection against the irreversible antagonist, chlor-natrexamine, to show that the receptor for dynorphin in the myenteric plexus of the guinea pig is distinct from that for morphine or Leu-enkephalin. Concurrent desensitization of δ and μ receptors in mouse vas deferens also showed that the dynorphin receptor is distinct from that for other opiates^{16,28}.

The effectiveness of dynorphin and Met-enkephalin as direct contractile agonists agrees with their relative abundance in the stomach and intestine and their preponderance in the circular muscle layer. Similar results were obtained recently in smooth muscle cells isolated from the human stomach and canine gall bladder (K.N.B. and G.M.M., unpublished observations). The finding of a direct effect favours a role for these peptides as contractile neurotransmitters. However, dynorphin and Met-enkephalin are equally effective neural opiate agonists in the gut. Enkephalinergic fibers ramify with other fibers in the circular muscle layer, forming axonal bundles that could well permit axo-axonal interactions such as presynaptic inhibition of ACh release from cholinergic nerve terminals. It is even possible that enkephalins and ACh co-exist in some nerve terminals of the myenteric plexus as they do in sacral preganglionic fibres in the viscera of the cat²⁹ and postganglionic cholinergic terminals in the adrenal medulla³⁰. Co-release, like adjacent release of enkephalins, could inhibit further presynaptic release of ACh.

Physiologically, the outcome is probably a combination of direct excitatory and indirect neurally-mediated inhibitory effects, the former inducing tonic motor activity in the gut, the latter, phasic or peristaltic activity³¹. The topography of enkephalin fibres in the gut indicates that a myenteric plexus-circular muscle preparation is the more apt physiological model with which to test neurally-mediated effects of enkephalin.

This work was supported by NIH grant AM-15564 and USPHS grant AM-28300.

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Opiate receptor subclasses differ in their conformational requirements

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Pharmacological and biochemical studies of classical opiates and opioid peptide analogues have revealed the existence of several subclasses of opiate receptors¹⁻⁵, among which the μ -, δ -, κ - and σ -receptors have been most widely discussed. The physiological roles of the different receptor classes and their structural characteristics remain to be elucidated. Recently, a cyclic analogue of enkephalin, H-Tyr-cyclo[-N^γ-D-A₂bu-Gly-Phe-Leu-] (Fig. 1, compound I; A₂bu represents α,γ -diaminobutyric acid), showing high potency in μ -receptor-selective bio- and binding assays has been described⁶. To assess the effect of the conformational constraint introduced by cyclization on opiate receptor selectivity, we have compared the cyclic compound I with its corresponding open-chain analogue, [D-Abu²,Leu⁵]enkephalinamide (Fig. 1, compound II; Abu represents α -aminobutyric acid), in bioassays based on inhibition of electrically evoked contractions of the guinea pig ileum and the mouse vas deferens, and in binding assays using μ - and δ -receptor-selective radiolabels. The differences in potency of the two compounds observed in the four assay systems suggest that the various opiate receptor subclasses have different preferences in terms of conformational properties of 'complementary' ligands.

Compared with [Leu⁵]enkephalin (H-Tyr-Gly-Gly-Phe-Leu-OH; III in Table 1)⁷, the cyclic analogue I shows a 17-fold increase in potency in the guinea-pig ileum (GPI)-assay and is twice as potent as the linear analogue, II (Table 1). The effect of both analogues on GPI was found to be naloxone-reversible. In the case of compound I, the apparent dissociation constant (K_d) obtained with naloxone as antagonist was 12 times lower than that determined for [Leu⁵]enkephalin and comparable to that observed for the μ -receptor-selective agonist, levorphanol. These observations indicate that the cyclic analogue exerts its effect on the GPI via interaction with μ -receptors.

In contrast to the results of the GPI assay, however, the cyclic analogue I is seven times less potent than the naturally occurring

Received 19 October 1981; accepted 23 February 1982.

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Table 1 Inhibitory potencies (IC_{50} s) and sensitivities to naloxone (K_e) of peptide analogues, [Leu^5]enkephalin and levorphanol in the guinea pig ileum and mouse vas deferens assays

No.	Compound	IC_{50} (nM)*		IC_{50} (MVD)/ IC_{50} (GPI)	K_e (nM)*	
		GPI	MVD		GPI	MVD
I	Tyr-cyclo[- N^{γ} -D-A $_2$ bu-Gly-Phe-Leu-]	14.1 $\pm$ 2.9	81.4 $\pm$ 5.8	5.77	0.122 $\pm$ 0.028	0.576 $\pm$ 0.044
II	[D-Abu 2 ,Leu 5]enkephalinamide	28.7 $\pm$ 1.3	45.6 $\pm$ 9.1	1.59	0.181 $\pm$ 0.033	1.50 $\pm$ 0.19
III	[Leu 5]enkephalin	246 $\pm$ 39	11.4 $\pm$ 1.1	0.046	1.53 $\pm$ 0.43	5.86 $\pm$ 0.90
IV	Levorphanol	17.0 $\pm$ 3.0	278 $\pm$ 56	16.4	0.064 $\pm$ 0.005	0.837 $\pm$ 0.182

The assay based on inhibition of electrically induced contractions of the GPI 19 was performed as described elsewhere 20 . The MVD assay was performed as described previously 21 . A log dose-response curve was determined using [Leu^5]enkephalin as the standard for each ileum or vas deferens preparation and IC_{50} s of the compounds being tested were normalized according to a published procedure 22 . K_e values for naloxone as antagonist were determined from the ratio (DR) of IC_{50} values obtained in the presence and absence of a fixed naloxone concentration (a), using the equation $K_e = a/(DR - 1)^{23}$.

The chemical syntheses of analogues I and II are described elsewhere 6,17 .

* Mean of three determinations ($\pm$ s.e.m.).

peptide and half as potent as its corresponding open-chain analogue (II) in the mouse vas deferens (MVD)-assay. There is evidence that, in addition to the predominant δ -receptor, the MVD also contains μ - and κ -receptors. As indicated by the high naloxone K_e (5.86 $\pm$ 0.90 nM), [Leu^5]enkephalin interacts selectively with δ -receptors in the MVD preparation, whereas levorphanol, with a low K_e value of 0.837 $\pm$ 0.182 nM, has high preference for μ -receptors in this tissue. The μ -receptor preference of the cyclic analogue I is revealed by a similarly low K_e (0.576 $\pm$ 0.044 nM), whereas analogue II shows a K_e (1.50 $\pm$ 0.19 nM) intermediate between those of levorphanol and [Leu^5]enkephalin, indicating its mixed $\mu + \delta$ character.

The MVD/GPI IC_{50} (50% inhibitory concentration) ratio observed for the cyclic analogue I was 125 times higher than that of [Leu^5]enkephalin and was comparable to the ratios determined for the μ -type agonists, [D-Ala 2 ,MePhe 4 ,Met(O)-ol 5]enkephalin 8 and [D-Ala 2 ,MePhe 4 ,Gly-ol 5]enkephalin 9 . Interestingly, the cyclic compound I also showed a MVD/GPI IC_{50} ratio about four times higher than the open-chain analogue II. This result, together with the difference in the naloxone K_e values observed in the MVD assay, suggests that the conformationally restricted analogue I has a lower preference for δ -receptor than the more flexible open-chain analogue II.

Affinities for opiate receptors in rat brain membrane preparations were determined in parallel binding assays using 3H -naloxone as a radioligand which preferentially binds to μ -receptors and 3H -[D-Ala 2 ,D-Leu 5]enkephalin as a radiolabel with preference for δ -receptors (Table 2). Sodium ratios of 47.0 and 38.9 were determined for analogues I and II, respectively, from 3H -naloxone displacement curves obtained in the absence and presence (100 mM) of sodium chloride. This observation indicates the agonist character of both compounds 10 . In the δ -receptor-selective binding assay, analogue I showed a 28-fold decrease in affinity compared with analogue II, whereas in the μ -receptor-selective binding assay, only a twofold reduction in affinity was observed. The nearly equal affinity of the linear peptide II for μ - and δ -receptors and the low affinity of the cyclic compound I for δ -receptors are reflected in the ratios of the inhibition constants (K_i^{δ}/K_i^{μ}) obtained from the two binding assays. We observed some discrepancies between the binding data and the results of the bioassays; analogue I was twice as potent as its linear counterpart II in the GPI assay, whereas in the 3H -naloxone binding assay, almost the reverse was true. A divergent effect of the conformational constraint in I on 'efficacy' and affinity represents one possible explanation for this observation. Cyclization would reduce affinity for μ -receptors but lead to a more productive peptide-receptor complex. On the basis of the well-known correlation between sodium ratio and 'efficacy' 10 , the higher sodium ratio observed for analogue I compared with II seems to support this hypothesis. Alternatively, a third type of receptor may be involved and the simple μ/δ -receptor concept may be insufficient to explain the present data. That the situation in rat brain may indeed be more complicated is indicated

by the recent demonstration 5 of three major types of morphine and enkephalin receptors (μ_1 , μ_2 and δ). Finally, it is interesting to note that cyclization produces only a twofold potency decrease in the MVD assay but a 28-fold affinity decrease in the δ -receptor-selective binding assay. This apparent discrepancy can be explained by the fact that the greatly reduced affinity of the cyclic analogue for δ -receptors is partly compensated by its ability to act as a highly productive μ -agonist in the MVD, as indicated by its low naloxone K_e .

Thus, comparison of analogues I and II in bioassays and binding assays reveals that introduction of the conformational constraint by ring closure in analogue I is directly and uniquely responsible for its decreased δ -character compared with the linear peptide II. Obviously, the semi-rigid 14-membered ring structure of I is highly compatible with the topography of the μ -receptor, but causes a decrease in affinity for δ -receptors compared with the open-chain analogue II. Analysis of the conformational possibilities of I revealed that several conformations proposed for native enkephalin in solution or when bound to its receptor cannot be adopted by the cyclic analogue 6 .

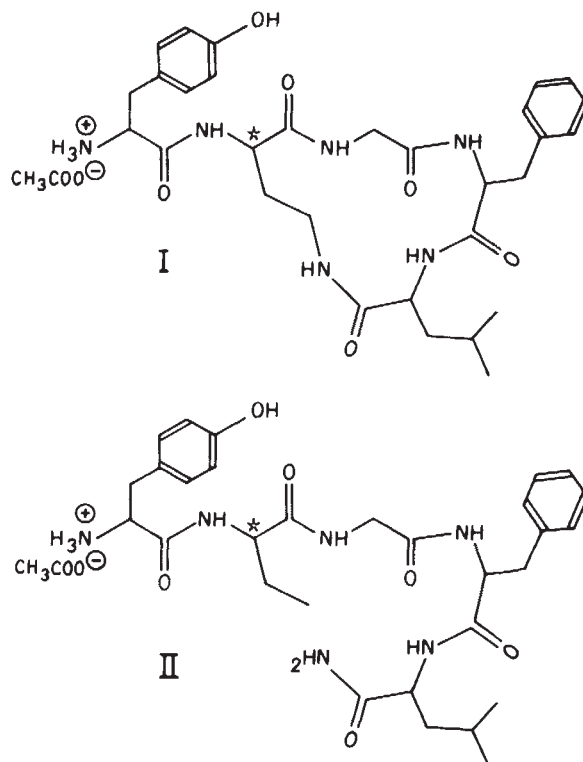


Fig. 1 Structural formulas of the cyclic enkephalin analogue, Tyr-cyclo[- N^{γ} -D-A $_2$ bu-Gly-Phe-Leu-] (I), and its corresponding open-chain analogue, [D-Abu 2 ,Leu 5]enkephalinamide (II).

Table 2 Inhibitory effects of peptide analogues and [Leu⁵]enkephalin on the binding of ³H-naloxone and ³H-[D-Ala², D-Leu⁵]enkephalin in rat brain homogenates

No.	Compound	³ H-naloxone binding, K _i ^a (nM)	³ H-[D-Ala ² , D-Leu ⁵]enkephalin binding, K _i ^a (nM)	K _i ^b /K _i ^a
I	Tyr-cyclo[-N ^γ -D-Ala ² -Gly-Phe-Leu-]	13.8 ± 3.4	115 ± 16	8.33
II	[D-Abu ² , Leu ⁵]enkephalinamide	5.60 ± 0.11	4.06 ± 1.46	0.725
III	[Leu ⁵]enkephalin	27.7 ± 5.7	8.05 ± 0.96	0.291

Binding studies using rat brain membrane preparations were carried out as described elsewhere²⁰. ³H-naloxone and ³H-[D-Ala², D-Leu⁵]enkephalin at concentrations of 0.40 and 0.96 nM, respectively, were used as radioligands and incubations were performed at 0°C for 1 h. K_i values were calculated based on Cheng and Prusoff's equation²⁴ using values of 0.5 and 0.8 nM for the dissociation of ³H-naloxone and ³H-[D-Ala², D-Leu⁵]enkephalin, respectively. Values shown are the mean of three determinations (±s.e.m.).

As the open-chain analogue is distinguished from the cyclic compound merely by the opening of a single carbon–nitrogen bond (Fig. 1), these results permit the unambiguous conclusion that different opiate receptor subclasses have different conformational requirements. The difference in receptor topography could be due to the existence of different conformational forms of a single opiate receptor protein, the distribution of which might be regulated by allosteric effectors¹¹. Alternatively, heterogeneity in opiate receptor topography could be due to minor changes in receptor protein sequence¹².

The results described here represent the first direct demonstration of a difference in conformational requirements between receptor subclasses in a situation of peptide receptor heterogeneity. In the case of somatostatin^{13,14} and bradykinin¹⁵, differences in receptor topography between receptor types mediating different biological effects have been suspected but could not be demonstrated unambiguously, because in these studies, based on pharmacological profiles of peptide analogues, the effect of amino acid substitutions could not be dissociated from a possible conformational effect. Evidence for a difference in conformational requirements between different receptor subclasses has been obtained in the case of γ-aminobutyric acid¹⁶.

Most linear peptides occurring in nature are very flexible and therefore able to adapt to different receptor surfaces without showing much selectivity. The demonstrated existence of receptor subclasses with topographically distinct features opens up an avenue for the design of receptor-selective peptide ligands by incorporation of appropriate conformational constraints. Subtle variation of the conformational restriction by modification of the ring size in analogue I¹⁷ or by means of a different type of cyclization¹⁸ has resulted in cyclic enkephalin analogues displaying various degrees of receptor selectivity.

We thank C. Lemieux and T. M.-D. Nguyen for technical assistance. This research was supported by an operating grant from the MRC of Canada (MT-5655).

Received 8 December 1981; accepted 16 March 1982.

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Dopamine modulates a Ca²⁺-activated potassium conductance in mammalian hippocampal pyramidal cells

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Dopamine (DA) is a neurotransmitter in the mammalian central nervous system which has proven or potential importance in such neurological disorders as parkinsonism, Huntington's chorea and epilepsy. Most of the electrophysiological data concerning the actions of DA in the brain have been obtained from studies in the caudate nucleus where DA produces neuronal depolarization and increased spike discharge¹, slow depolarization with decreased spike discharge^{2,3} and an increase in apparent input resistance³ and hyperpolarization with reduced firing rate³. The mechanisms underlying these effects have not been examined. The evidence suggests that the hippocampus receives a dopaminergic projection^{4–6} and that DA inhibits most hippocampal neurones^{7,8}. We have studied the effects of DA on CA1 hippocampal pyramidal cells (HPCs) *in vitro* and report here that DA causes prolonged inhibition associated with hyperpolarization and increased conductance. These effects seem to derive from induction of a Ca²⁺-activated K⁺ conductance, and would make DA effective in modulating the high frequency firing and burst generation which occurs normally in some neurones^{9,10}, and pathologically in HPCs during epileptogenesis¹¹.

Hippocampal slices were prepared and maintained as previously described¹². Intracellular recordings were obtained with K-acetate (4 M)-filled microelectrodes having resistances of 25–60 MΩ. Freshly prepared DA (10^{–6}–10^{–4} M) in standard Ringer's solution (pH 7.4)¹² was applied either by pressure ejection of droplets from a broken micropipette on to dendritic areas of CA1 HPCs¹³ or via superfusion (10^{–5} M); these two methods produced identical results. Neurones having stable resting membrane potentials > –55 mV for longer than 5 min, and action potential amplitudes > 70 mV were selected for study. Current was delivered through an active bridge circuit and bridge balance which was constantly monitored and adjusted. Control data were obtained with electrodes in the extracellular position so that records from micropipettes which showed polarization artefacts or rectification could be disregarded. Membrane potential (V_m) was determined by assuming the bath potential to be 0 mV.

Changes due to DA application were observed in 16 of 21 CA1 HPCs; in 13 of these, membrane properties were characterized in detail before and after drug application. The initial mean input resistance (R_N) of this group of cells was 38.9 ± 10.7

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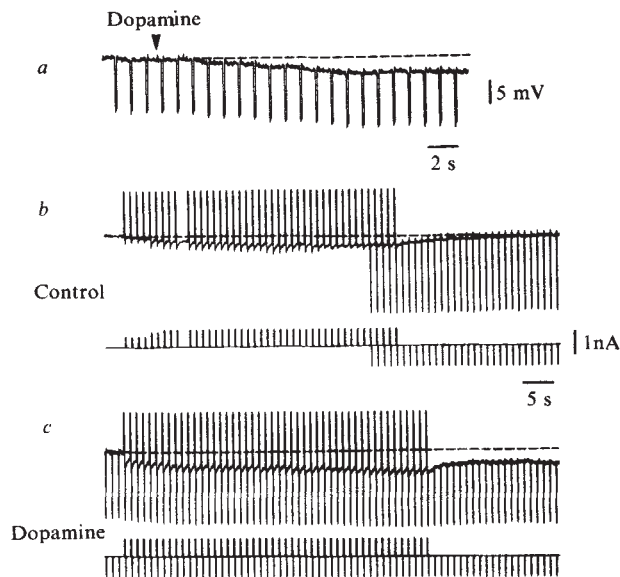


Fig. 1 Examples of DA-induced hyperpolarizations. *a*, Chart recording showing the hyperpolarizing response of a cell to application of 10 μ M DA. The voltage response to constant-current hyperpolarizing pulses shows that no detectable change of input resistance (R_N) accompanies this hyperpolarization. Resting potential, -60 mV. *b*, In another neurone in normal conditions, a 1-Hz train of 120-ms depolarizing current pulses, of sufficient intensity to elicit 2–10 spikes, results in membrane hyperpolarization associated with a decrease in R_N . When depolarizing pulses are discontinued the membrane repolarizes and resistance recovers to the control level. *c*, After DA application to the cell in *b*, the membrane hyperpolarized by a few millivolts (data not shown). The membrane potential was then depolarized to the control level with d.c. current and a train of depolarizing pulses was delivered comparable to that in *b*. R_N decreased during the train. Membrane potential and resistance at the end of the train of depolarizing pulses did not recover to pre-stimulus levels. Control resting potential for the neurone in *b* and *c* was -56 mV. Time and current calibrations in *b* apply to *c* also. Voltage calibration in *a* applies to *b* and *c*. Spikes were amputated by inkwriter. Lower traces in *b*, *c*: current monitor, depolarizing current up. Broken lines represent baseline resting potentials.

($\pm$ s.d.) M Ω at a mean resting potential of -59.7 ± 5.7 mV. These values are similar to those previously reported¹³. DA application caused a small initial hyperpolarization (mean amplitude 4.9 ± 3.6 mV) in 10 of 13 cells within 3–40 s of delivery (Fig. 1*a*). Often this was not associated with a prominent change in R_N , although decreases in R_N were seen in cells which showed the largest hyperpolarizing responses, or when additional doses of DA were applied to produce further hyperpolarization. The shift in V_m was often sufficient to decrease firing frequency or silence active discharge. Further hyperpolarization was triggered by 120-ms depolarizing current pulses which evoked spike trains. It is known that slow after-hyperpolarizations (AHPs) lasting up to 1–2 s normally follow repetitive spike discharges in HPCs^{9,10,14,15}. We found that, in control conditions, when depolarizing current pulses were delivered at frequencies of ~ 1 Hz, and at sufficient intensity to elicit 2–10 action potentials, these slow AHPs summated until steady membrane potential was reached (Fig. 1*b*). In the control state, V_m consistently recovered to the baseline level within seconds after such stimulation was discontinued. Following DA application, however, the slow AHPs evoked by the same depolarizing current pulses were larger and there was no recovery of V_m to the baseline potential after cessation of the pulses (Fig. 1*c*). In addition, slow AHPs following a single evoked spike train (tested at control V_m maintained with d.c. current) were also augmented (Fig. 2*a–c*). Such AHPs were of larger amplitude than those in control neurones and lasted for as long as 18 s (13 s in the case of Fig. 2*d*). Augmented AHPs were also found in neurones impaired after DA application (see below). The prolonged duration was not dependent on the passage of intracellular d.c. current. The DA effect on AHPs was detectable in seconds and reached a maximum after several

minutes. In addition it appeared that the actions on the AHP were due to some cumulative process initiated by spike trains, as a sequence of conditioning trains evoked at 1 Hz by 120-ms depolarizing pulses increased the duration of an AHP triggered seconds later.

The maximum total hyperpolarization, that is, that which occurred after DA delivery (Fig. 1*a*) plus that which could be further evoked during summated AHPs (Fig. 1*c*), was 7.7 ± 6.0 mV ($n = 13$). These peak increases in V_m were associated with decreases in R_N of 19–42% (compare amplitude of responses to hyperpolarizing pulses in Fig. 2*a*, *b*, before and after DA). The changes in R_N which accompanied the DA-induced hyperpolarization were not related to membrane rectification: for

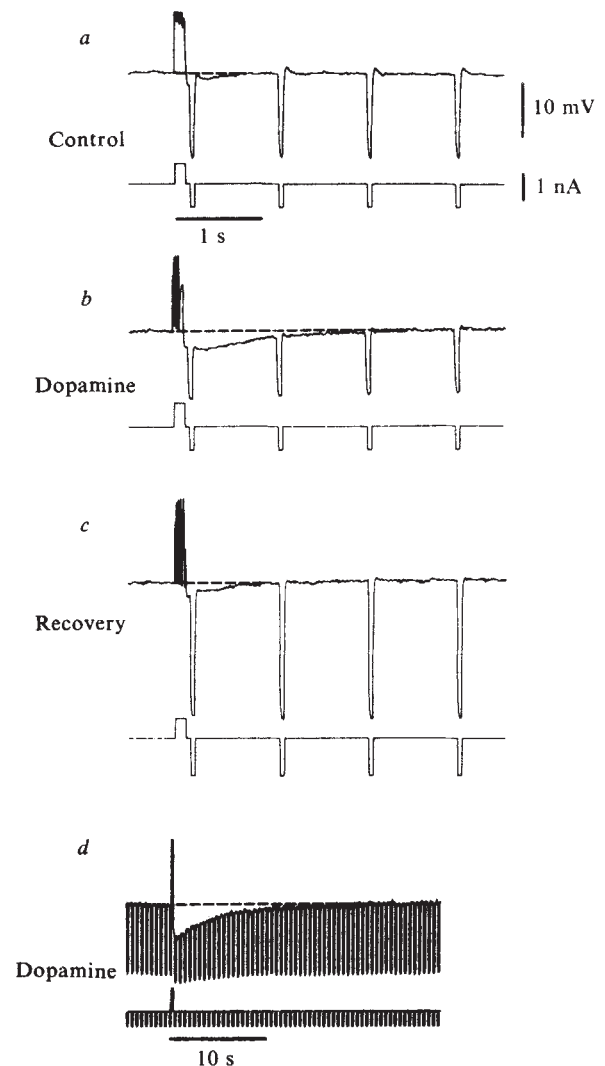


Fig. 2 Slow AHPs induced by depolarizing current pulses. *a*, Chart recording of control response to a depolarizing pulse. The AHP lasts 600 ms, reaches 2 mV in amplitude, and is associated with a peak decrease in resistance of $\sim 11\%$ (ref. 14). *b*, Following DA application (10 μ M) this cell hyperpolarized (not shown) and input resistance decreased. The membrane was depolarized to the resting potential of *a* and the amplitude of the depolarizing current pulse adjusted to elicit a number of spikes comparable to those of the stimulus in *a*. This was necessary because of a decrease in baseline R_N of $\sim 35\%$ compared with the control. In these conditions AHPs lasted for 2.5 s, reached 4 mV in amplitude, and were associated with proportionately larger decreases in resistance (peak decrease of $\sim 20\%$). The apparent difference in responses to initial depolarizing pulses in *a* compared with *b* is due to distortion produced by the low frequency response of the chart recorder, and the effect of DA in decreasing R_N . *c*, Recovery of AHPs to control duration and amplitude was seen in this same neurone 45–60 min after DA application. *d*, Much longer duration AHPs (> 10 s) and associated resistance changes were evoked in several cells. In this neurone, impaled 1 h after DA application near the cell of *a–c*, the AHP lasted for 13 s. No d.c. current was passed through the electrode during this recording. Voltage and current calibrations in *a* apply to *b–d* also. Time calibration in *a* applies to *b* and *c*.

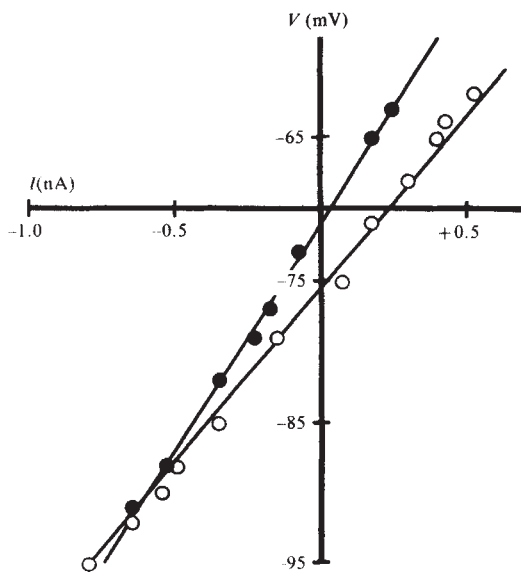


Fig. 3 Representative current-voltage relationship in one cell before (●) and after (○) DA application (10 μ M). Curves were obtained by plotting steady-state applied pipette current against the membrane potential. Current (I)-voltage (V) lines were drawn using linear regression to obtain a least-squares fit of the data. The reversal potential for DA hyperpolarization in this neurone, determined from the point of intersection of these curves, was -91 mV.

example, the mean R_N before DA application was 37.3 ± 10.2 M Ω at the original resting V_m of -60.1 ± 6.0 mV ($n = 11$). After peak DA-induced hyperpolarization was attained, sufficient d.c. current was delivered to depolarize HPCs back to the original resting V_m . The mean R_N measured at this voltage level was 29.0 ± 8.8 M Ω , which was a significant ($P < 0.005$) decrease of 22.2% from control values.

A reversal potential for the DA hyperpolarization was obtained from plots of voltage against applied current before and after DA application. Figure 3 shows a representative plot from one neurone. The mean reversal potential obtained from intersection of voltage-current lines was -86.8 ± 11.1 mV ($n = 8$), a value consistent with DA increasing K^+ conductance (g_K)¹⁵, and more negative than the expected Cl^- equilibrium potential of ~ -70 mV (ref. 16). This conclusion was supported by the observation that DA-induced increases in V_m were equally prominent in neurones impaled with KCl-containing microelectrodes, even when inhibitory postsynaptic potentials (i.p.s.ps), known to be primarily Cl^- -mediated^{17,18}, were reversed.

In most cases, recovery from DA-induced hyperpolarization or augmentation of AHPs did not occur during a single impalement. However, in one particularly stable recording, it was possible to follow the time course of DA influence over a period of 2 h (Fig. 2). During the control period, AHPs reached a peak amplitude of 2 mV, and lasted ~ 600 ms (Fig. 2a). Following focal application of DA and adjustment of V_m to the control level with d.c. current, the AHP peak amplitude increased to 4 mV and lasted 2.5 s (Fig. 2b); recovery occurred in 45 min (Fig. 2c). Other neurones in the same area impaled up to 1 h after DA application showed similar or even more pronounced effects (Fig. 2d). No d.c. current was passed to manipulate V_m in cells impaled after DA application since resting V_m before DA application was unknown. The AHP durations and amplitudes, however, were significantly larger and longer than those found in control neurones in these and previous experiments¹⁴. These data suggest that the DA-induced alterations are reversible, but long-lasting.

Our results are similar in some respects to those reported by Herrling⁸ for anaesthetized cats. In both cases cell discharges were decreased or blocked, and in most cells a hyperpolarization developed. However, Herrling found an increase in resistance

in four neurones tested, in contrast to our results. The reasons for this discrepancy are not clear, but could relate to the action of anaesthetic or the technical difficulties in studying partially depolarized neurones *in vivo*.

The most reasonable explanation for the DA-induced hyperpolarization and the augmentation of AHPs is that they both derive from increases in K^+ permeability. As noted above, the equilibrium potential for DA hyperpolarization is in the range expected for this ion (Fig. 3). Effects on AHPs could be due to enhancement of the Ca^{2+} -activated g_K which underlies these events in HPCs^{14,15}. DA-enhanced and normal AHPs are associated with conductance increases, have reversal potentials at very negative levels, and may both be blocked by Mn^{2+} , a Ca^{2+} antagonist^{14,26}. If the DA-enhanced slow AHP were due in part to Na^+-K^+ pump activation¹⁹, we would have expected persistence of at least a component of this potential when trains of spikes were evoked in Mn^{2+} solutions. Although all slow AHPs were blocked in 3 mM Mn^{2+} solutions, hyperpolarizations associated with conductance increases similar to that shown in Fig. 1a were still observed after DA application to some neurones. These effects must be postsynaptic as this concentration of Mn^{2+} blocks synaptic transmission in hippocampal slices¹⁴.

Effects of DA in inducing or enhancing a Ca^{2+} -dependent K^+ conductance could result from effects on processes which increase intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) as proposed for neocortical neurones following dinitrophenol injection²⁰ and for cockroach neurones after DA application²¹. For example, the DA-induced initial hyperpolarization could result from release of Ca^{2+} from intracellular stores, or decreased Ca^{2+} buffering. These actions, combined with tonic Ca^{2+} entry²², would prolong and increase K^+ conductance. Alternatively, DA may enhance calcium entry into neurones—this is suggested by its reported effects in cardiac muscle fibres²³; however the persistence of DA-induced hyperpolarization in Mn^{2+} solutions that block Ca^{2+} entry²⁶ is more consistent with a DA-mediated release of Ca^{2+} from intracellular stores. The cumulative hyperpolarization which follows repetitive stimulus-induced spike trains (for example, Fig. 1c) would thus derive from prolonged increases in $[Ca^{2+}]_i$ following Ca^{2+} influx, which in turn would activate a sustained change in K^+ conductance. Recent experiments showing that EGTA injection into neurones blocks DA-induced hyperpolarization and DA-enhanced AHPs further substantiate the involvement of Ca^{2+} in DA effects²⁶.

DA is a potent inhibitory agent which modulates the relationship between cell activity and the development of intrinsic inhibitory membrane properties in HPCs through effects on the Ca^{2+} -activated K^+ conductance. Its inhibitory action would be most effective during periods of Ca^{2+} entry associated with burst generation¹⁰ and high frequency firing such as occur in CA1 HPCs following exposure to acetylcholine¹³, and during epileptogenesis^{11,15,24}. The possibility that DA modulates such burst generation and seizure susceptibility *in vivo* is supported by anatomical data demonstrating a more or less direct projection of the hippocampal complex to the cells of origin of the dopaminergic projection²⁵.

We thank Drs B. W. Connors, L. M. Masukawa, B. R. Ransom and S. H. Thompson for helpful discussion, Ms C. Joo for secretarial assistance, and Mr J. L. Kadis for technical help. This work was supported by NS 12151 and NS 06477 (D.A.P.), a NSF Graduate Fellowship (L.S.B.), and the Morris Research Fund.

Received 23 July 1981; accepted 2 March 1982.

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Lipid-soluble toxins thought to be specific for Na⁺ channels block Ca²⁺ channels in neuronal cells

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Many toxic compounds alter specifically the functioning of the fast sodium conductance that is responsible for the generation of action potentials in neuronal and muscle cells. One important class of these compounds consists of the lipid-soluble toxins, such as veratridine and other ceveratrum alkaloids, batrachotoxin, aconitine, and the diterpenoid grayanotoxins. These molecules are thought to bind to a common receptor site on the Na⁺ channel^{1–5}, thus inducing a membrane depolarization which can be suppressed by the addition of tetrodotoxin (TTX) or by the removal of Na⁺ from the incubation medium³. We show here that this family of molecules is not specific for the Na⁺ channel in neuroblastoma cells. Blockade of the voltage-dependent calcium channel is also observed, either in the toxin concentration range in which the molecules act on the Na⁺ channel (veratridine, grayanotoxin) or at even lower concentration (batrachotoxin).

Voltage-clamp experiments on differentiated N1E 115 neuroblastoma cells have revealed the existence of two voltage-dependent transient inward currents: a fast Na⁺ current that is TTX-inhibitable and a slow Ca²⁺ current showing a voltage- and time-dependent inactivation⁶. Similarly to Ca²⁺ channels described in other preparations, the Ca²⁺ channel of neuroblastoma cells has the following properties: it is permeable to Ba²⁺

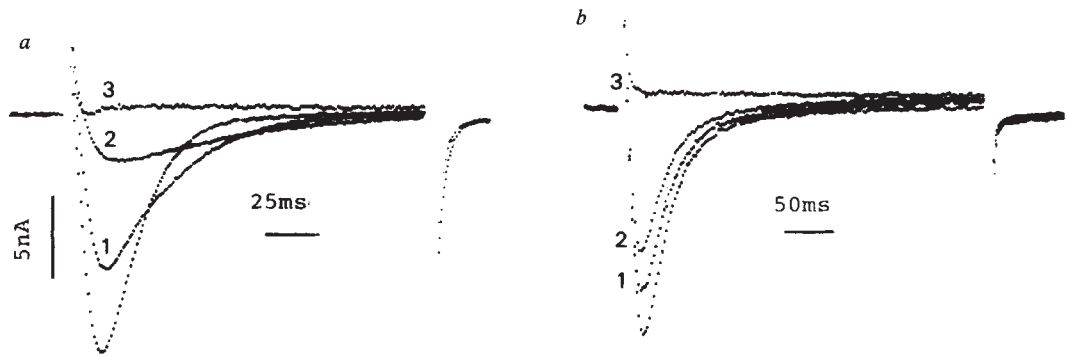
and Sr²⁺ cations⁶, is inhibited by Mn²⁺, Co²⁺, Ni²⁺ or La³⁺ cations⁶, is unaffected by TTX⁶, and is blocked by the so-called Ca²⁺ channel blockers, verapamil or D₆₀₀, but only at concentrations as high as 0.1 mM (G.R., unpublished results). Voltage-clamp experiments presented in Fig. 1 show that veratridine and batrachotoxin cause a progressive decrease and ultimately a complete block of the Ca²⁺ current. The following are the main differences between the effects of veratridine and batrachotoxin: (1) batrachotoxin blocks the Ca²⁺ channel at a much lower concentration than veratridine. This difference will be analysed in more detail below. (2) The decrease of Ca²⁺ current due to the action of veratridine (Fig. 1a) is accompanied by a slowing of the inactivation step, whereas the kinetics of the Ca²⁺ channel are unchanged in the presence of batrachotoxin (Fig. 1b). (3) Veratridine blockage of the Ca²⁺ channel is rapidly reversible on washing for a few minutes (Fig. 2) whereas washing for more than 1 h does not remove the effects of batrachotoxin.

The peak amplitude of transient Ca²⁺ currents before and after applications of 20 and 200 µM veratridine is plotted as a function of membrane potential in Fig. 3a. Veratridine blockade of Ca²⁺ channels is partial at 20 µM and essentially complete at 200 µM, and is accompanied by a shift of the current-voltage curve towards more negative membrane potentials. Figure 3b shows dose-response curves for the action of veratridine and batrachotoxin in suppressing the transient Ca²⁺ current. The half-maximum effects (*K*_{0.5}) of the toxins are observed at concentrations of 26 µM and 40 nM for veratridine and batrachotoxin, respectively. Cevadine and α-dihydrograyanotoxin II are active in the same concentration range as veratridine.

The concentrations of veratridine and α-dihydrograyanotoxin II required for half-maximal stimulation of ²²Na⁺ influx in the same neuroblastoma cells were 44 and 90 µM, respectively^{7,8}. Thus, veratridine and α-dihydrograyanotoxin II act on both Ca²⁺ channels and Na⁺ channels in the same range of concentration. In contrast, the *K*_{0.5} value for the action of batrachotoxin on the neuroblastoma Na⁺ channel is ~1 µM (ref. 8), which is about an order of magnitude larger than that found for the action of the toxin on Ca²⁺ channels.

Dose-response curves for the action of veratridine, cevadine, α-dihydrograyanotoxin II or batrachotoxin on Na⁺ channels in neuroblastoma cells have been obtained in the presence of 1.8 mM external Ca²⁺. However, higher Ca²⁺ concentrations strongly antagonize this effect of these lipid-soluble toxins^{3,5} and it is largely suppressed at 25 mM Ca²⁺, the concentration at which Ca²⁺ currents were recorded here. Therefore, at high external Ca²⁺ concentrations, veratridine, α-dihydrograyanotoxin II and batrachotoxin seem to be much more specific for Ca²⁺ channels than for Na⁺ channels.

Fig. 1 Voltage-clamp analysis of the effects of veratridine (a) and batrachotoxin (b) on the Ca²⁺ current of N1E 115 neuroblastoma cells. Depolarizing pulses of 70 mV amplitude and 170 ms duration were applied from a holding potential of -80 mV. a, Time course of the effect of 2 × 10⁻⁴ M veratridine on the Ca²⁺ current. Successive recordings were taken 1 (1), 2 (2) and 5 (3) min after addition of veratridine. b, Time-course of the effect of 2 × 10⁻⁷ M batrachotoxin on the Ca²⁺ current. Successive recordings were taken 10 (1), 15 (2) and 30 (3) min after addition of batrachotoxin. Culture dishes containing N1E 115 neuroblastoma cells were used directly after the culture medium had been replaced by a Na⁺-free solution containing 25 mM CaCl₂, 0.4 mM MgSO₄, 5.4 mM KCl, 25 mM HEPES-Tris, 5 mM glucose and 25 mM tetraethylammonium, adjusted to pH 7.4 and to an osmotic pressure of 305 mosmol with choline chloride. Tetraethylammonium was used to block the delayed outward K⁺ current, which partially masked the Ca²⁺ current⁶. Voltage-clamp experiments were performed with the use of a suction pipette method analogous to that described by Lee *et al.* for snail neurones¹⁰. The intracellular perfusion solution was 10 mM NaH₂PO₄ and 115 mM glutamic acid, adjusted to pH 7.1 with KOH and to an osmotic pressure of 305 mosmol with sucrose.



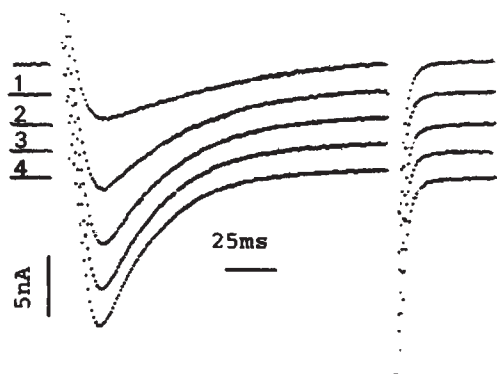


Fig. 2 Reversal by washing of the blocking effect of 10^{-4} M veratridine on the Ca^{2+} current. Successive recordings were taken 1 (1), 2 (2), 3 (3) and 4 (4) min after the beginning of washing. The recovered Ca^{2+} current in (4) was reduced by $\sim 10\%$ from the control Ca^{2+} current measured before the application of veratridine.

Aconitine and ceveratrum alkaloids of the veratridine family such as germitrine, germitridine, germine-3-acetate and protoveratrine A, which cause a persistent activation of Na^{+} channels in neuroblastoma cells with an efficiency similar to that of veratridine (P. Honerjäger, C. Frelin and M. Lazdunski, in preparation), are inactive on Ca^{2+} channels, even at high concentrations (0.1 mM). Moreover, none of these molecules antagonizes the action of veratridine on the Ca^{2+} channel, so that they can be considered to be specific for the Na^{+} channels.

Other toxins that act on the Na^{+} channel include TTX and saxitoxin, and polypeptide toxins extracted from some scorpion venoms or sea anemones. TTX and saxitoxin block the increase of Na^{+} permeability produced either by electrical stimulation or by the lipid-soluble toxins. Polypeptide toxins specifically slow the inactivation of the Na^{+} channel without changing the properties of the activation step⁴. The polypeptide toxins act in synergy with the lipid-soluble toxins. In neuroblastoma cells they produce an increase in the fraction of Na^{+} channels activated at a saturating concentration of the lipid-soluble toxins⁷. We have shown that high concentrations of TTX (10 μM), of *Androctonus australis* Hector scorpion toxin II (0.1 μM) and of *Anemonia sulcata* toxin II (10 μM), are without effect on Ca^{2+} channels in neuroblastoma cells. Moreover, the same high concentrations of these toxins had no effect on the blockade of the Ca^{2+} channels caused by veratridine, regardless of whether they were added before or after the lipid-soluble toxin.

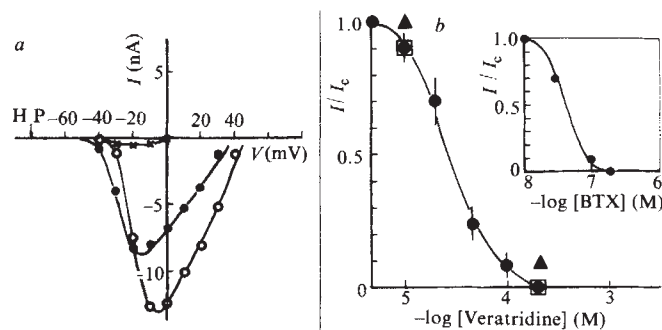


Fig. 3 *a*, Current-voltage relationships for the peak transient Ca^{2+} current obtained from the same cell before (○) and after a 10-min application of 2×10^{-5} M veratridine (●). Veratridine was then added at a final concentration of 2×10^{-4} M for 5 min (×). *b*, Effect of different concentrations of veratridine (●), cevadine (□), α -dihydrograysanotoxin II (▲) and batrachotoxin (inset) on the Ca^{2+} current. For each concentration of toxin, a depolarizing pulse of 70 mV was applied from a holding potential of -80 mV. I_c is the peak value of the Ca^{2+} current in the control, and I is the peak value in the presence of different amounts of toxin. For veratridine, each point represents the mean ratio of I/I_c ($\pm$ s.d.) from five experiments.

The present observation that veratridine and batrachotoxin block Ca^{2+} channels may lead to a reinterpretation of some of the results obtained by various researchers who used these toxins as tools on the assumption that they were specific for the Na^{+} channel.

The fact that veratridine, batrachotoxin, cevadine and α -dihydrograysanotoxin II act on both Na^{+} and Ca^{2+} channels suggests that some common structural feature may be necessary for the operation of both types of channel. As tritiated batrachotoxin has now been synthesized⁹ and as batrachotoxin is a very potent Ca^{2+} blocker, one of the most interesting possibilities for the future is that this toxin might become the first tool available for the biochemical titration of the Ca^{2+} channel.

Batrachotoxin was kindly provided by Dr J. Daly, α -dihydrograysanotoxin II by Drs M. Nakajima and Ryozi Irize and ceveratrum alkaloids by Dr P. Honerjäger. We thank Dr C. Frelin and M. T. Ravier for providing neuroblastoma cells and Dr Y. Tourneur for his collaboration. This work was supported by CNRS, DGRST and INSERM (CRL, 80.30.10).

Received 11 January; accepted 16 March 1982.

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Expression of eukaryotic coding sequences in *Methylophilus methylotrophus*

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Although there have been many reports of the expression of eukaryotic genes in *Escherichia coli* K-12 (refs 1-4), very little attention has focused on the expression of these sequences in other bacteria. The use of other organisms may prove advantageous, especially with regard to industrial processes. For example, work is in progress to develop alternative host/vector systems based on *Bacillus subtilis*⁵ and *Saccharomyces cerevisiae*⁶. The obligate methylotroph, *Methylophilus methylotrophus*, is a fermentation organism which grows very well on cheap substrates, methanol and ammonia. Moreover, obligate methylotrophs are unlikely to infect man and hence may be considered relatively safe. Here we report the expression of two eukaryotic cDNAs encoding chicken ovalbumin and mouse dihydrofolate reductase in *M. methylotrophus*, hence demonstrating the potential of this organism for the commercial production of mammalian peptides.

Most cloning vectors developed to date are plasmids or phages specific for *E. coli*. Wide host-range cloning vectors based on RP4 and other IncP group plasmids have been developed⁷, but these vectors are large, have a low copy number and are therefore not suitable for cloning when increased expression due to copy number is desired. For ease of manipulation and high gene dosage, small, high copy-number, broad

host-range plasmids are needed. IncQ group plasmids, including R300B⁸, meet these criteria and although they are themselves non-conjugative, they are mobilized efficiently by conjugative plasmids of various groups⁸. Despite its advantages, however, R300B has relatively few restriction enzyme sites suitable for cloning. The composite plasmid, pGSS15⁹, has both the ampicillin (Ap^r) and tetracycline (Tc^r) resistance from pBR322 while retaining the broad host-range characteristics of R300B and hence contains a number of unique restriction enzyme sites for cloning. Moreover, cloning of eukaryotic cDNA into the *Pst*I site of the Ap^r gene by dG.dC tailing has been demonstrated to lead to the expression of either fused¹⁰ or native¹¹ eukaryotic polypeptides at least in *E. coli*.

It has been reported previously that using broad host-range cloning vectors, the *E. coli* *gdh* gene has been expressed in *M. methylotrophus*¹². Here we have used pGSS15 to demonstrate expression of the mouse dihydrofolate reductase and chicken ovalbumin genes in *M. methylotrophus*.

Copy DNA derived from the mRNA for mouse dihydrofolate reductase (DHFR) has been cloned and expressed in *E. coli*¹³; bacteria which synthesize mouse DHFR can be selected because they are resistant to levels of trimethoprim that would ordinarily inhibit growth.

The plasmid pDHFR7 (ref. 13) was constructed by annealing deoxy-C-'tailed' cDNA to pBR322 DNA which had been cleaved in the β -lactamase gene by *Pst*I and 'tailed' with deoxy-G residues. The *Pst*I sites were regenerated on both ends of the insert as a result of such recircularization. The cDNA insert of pDHFR7 was isolated after *Pst*I cleavage of the plasmid and 1 μ g of this DNA was mixed with 0.5 μ g of pGSS15 DNA which had been linearized by *Pst*I digestion. The mixture was ligated and used to transform *E. coli* ED8654 (*metB*, *rk*⁻*mk*⁺, *supF*). Out of 221 of the trimethoprim-resistant (Tp^r) clones, 216 were ampicillin-sensitive, showing that the DHFR fragment had been inserted into the *bla* gene. Gel electrophoretic analysis showed that all the plasmids in the Tp^rAp^s clones were the same size and were larger than pGSS15. The DHFR cDNA has a single *Bgl*II restriction site which allows orientation of the fragment. *Eco*RI/*Bgl*II digests of plasmids from Tc^rAp^sTp^r and Tc^rAp^sTp^r clones established that only one orientation led to functional

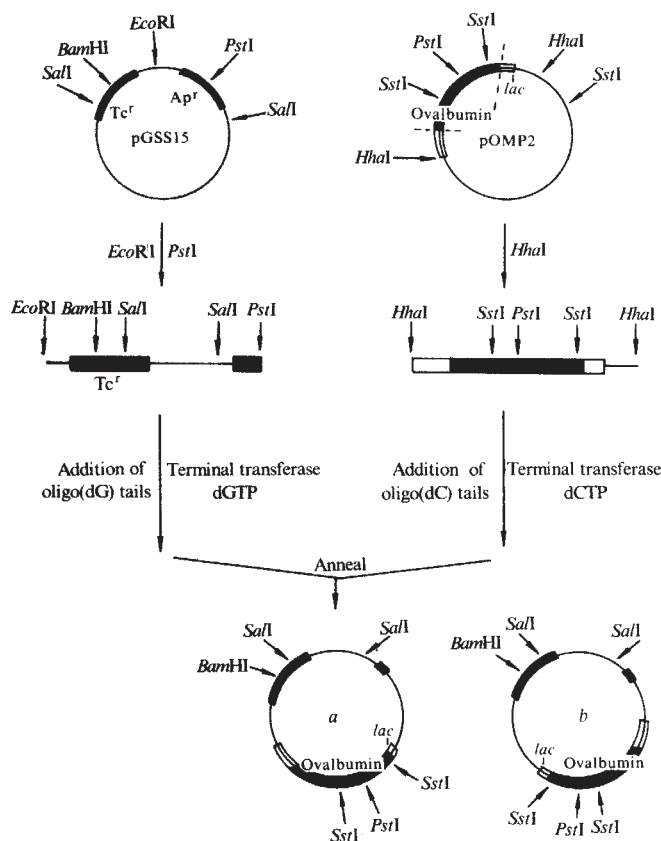


Fig. 1 Outline of the construction of pGSS15 derivatives carrying ovalbumin cDNA sequences fused to the *lac* UV5 promoter. pGSS15 DNA (1 μ g) was digested with *Eco*RI and *Pst*I and the larger fragment isolated from an agarose gel using hydroxyapatite²⁵. A *Hha*I DNA fragment containing the ovalbumin sequence and *lac* UV5 promoter from pOMP2 was isolated using the same technique. In the case of pOMP2, several other *Hha*I sites are present outside the *lac*-ovalbumin region (not shown). Oligo (dC) or (dG) tails were added using calf thymus terminal transferase, in the presence of 1 mM CaCl₂, and the two DNAs were mixed in equimolar amounts, at a total concentration of 1 μ g ml⁻¹, for annealing²⁶. Aliquots were used to transform CaCl₂-treated strain CL499 and transformants selected on nutrient plates containing (10 μ g ml⁻¹) tetracycline. Colonies were tested for the presence of ovalbumin DNA sequence by hybridization to the *Taq*I ovalbumin fragment labelled with ³²P, and finally for the production of immunoreactive ovalbumin.

Table 1 Dihydrofolate reductase activities of pDHFR 2.43 in extracts of *E. coli* ED 8654 and *M. methylotrophus*

Strain	Substrate	Addition	Activity
<i>E. coli</i> ED 8654	Dihydrofolate	—	0.70 ± 0.14 (4)
<i>E. coli</i> ED 8654 (pDHFR 2.43)	Dihydrofolate	—	1.58 ± 0.26 (5)
<i>M. methylotrophus</i>	Dihydrofolate	—	2.34 ± 0.68 (5)
<i>M. methylotrophus</i>	Dihydrofolate	Trimethoprim*	0 (2)
<i>M. methylotrophus</i>	Folate†	—	0 (2)
<i>M. methylotrophus</i> (pDHFR 2.43)	Dihydrofolate	—	12.6 ± 1.7 (4)
<i>M. methylotrophus</i> (pDHFR 2.43)	Dihydrofolate	Trimethoprim*	2.57 ± 0.53 (4)
<i>M. methylotrophus</i> (pDHFR 2.43)	Folate†	—	0.50 (1)

E. coli strains used were grown on medium containing (per l): (NH₄)₂SO₄, 2 g; K₂HPO₄, 14 g; KH₂PO₄, 6 g; trisodium citrate, 1 g; MgSO₄·7H₂O, 0.2 g (pH 7.2) supplemented with methionine (50 μ g ml⁻¹) and glucose added to a final concentration of 0.2%. *M. methylotrophus* strains were grown on methanol minimal medium as described previously¹². Cultures were grown with shaking at 37 °C overnight, collected by centrifugation and the pellets resuspended in 3 vol 50 mM potassium phosphate buffer (pH 7). The cells were disrupted by sonication on ice in the presence of 10 μ M phenylmethylsulphonyl fluoride (PMSF) and 10 μ M benzimidazole and cell debris removed by centrifugation at 10,000g for 15 min and 100,000g for 60 min. Enzyme assays were performed at 37 °C. The standard assay mixture contained 50 μ M dihydrofolate, 60 μ M NADPH, 12 mM 2-mercaptoethanol, 50 mM potassium phosphate buffer pH 7 and enzyme (normally 100 μ l of bacterial extract) in a final volume of 1.0 ml. The oxidation of NADPH was followed at 340 nm for ~10 min. Enzyme activity is expressed as nmol of NADPH oxidized per min per mg protein (mean ± s.e.m.). Numbers in parentheses are the number of separate experiments. Protein concentration was determined by the method of Lowry *et al.*²⁷.

* Trimethoprim (100 μ g ml⁻¹) was added to the enzyme assays.

† Folate (70 μ M) was included in the assay mixture instead of dihydrofolate.

expression of the DHFR sequence, indicating that productive transcription was from the β -lactamase promoter.

The plasmid R64drd11 (ref. 14) was introduced into one Tp^r clone, pDHFR 2.43, and used to mobilize this hybrid plasmid into *M. methylotrophus*. Table 1 shows the levels of dihydrofolate reductase activity in extracts of *E. coli* (pDHFR 2.43) and *M. methylotrophus* (pDHFR 2.43). When trimethoprim (100 μ g ml⁻¹) was added to the *M. methylotrophus* extracts, complete inhibition of the bacterial enzyme activity was observed, but the activity in the pDHFR 2.43 extract was reduced to only ~25%, consistent with observations that the mammalian enzyme is less prone to inhibition by trimethoprim¹⁵. Furthermore, changing the substrate to folate instead of dihydrofolate abolished the activity of the bacterial enzyme but the mammalian enzyme retained a low level of activity (Table 1 and ref. 16). These two observations confirm that the trimethoprim resistance of *M. methylotrophus* carrying pDHFR 2.43 is a consequence of the expression of the mouse dihydrofolate reductase gene.

As pGSS15 retains the broad host-range characteristics of R300B⁸, we have been able to transfer pDHFR 2.43 into another methylotroph, *Methylomonas methylotrova*, and into an autotrophic bacterium, *Alcaligenes eutrophus*, and have

Table 2 Synthesis of ovalbumin-like protein in bacterial extracts

Strain	Plasmid	Immunoreactive ovalbumin (molecules per cell)
<i>E. coli</i> CL499	pOV230	ND
<i>E. coli</i> CL499	pGSS15: <i>Taq</i> -ov	2.8
<i>M. methylotrophus</i>	pGSS15: <i>Taq</i> -ov	3.6
<i>E. coli</i> CL499	pOMP2	740
<i>E. coli</i> CL499	pGSS15: <i>lac</i> -ov	760
<i>M. methylotrophus</i>	pGSS15: <i>lac</i> -ov	150

Cultures (5 ml) of *E. coli* and *M. methylotrophus* were grown with shaking at 37 °C overnight. *E. coli* cultures were grown in Luria broth containing $10 \mu\text{g ml}^{-1}$ tetracycline and *M. methylotrophus* in methanol minimal medium¹² containing $1 \mu\text{g ml}^{-1}$ tetracycline. Cells were collected by centrifugation and washed in 5 ml of phosphate-buffered saline (PBS). The cells were resuspended in 5 ml of ice-cold PBS and disrupted by sonication. Cell debris was removed by centrifugation at 3,000g for 10 min and the cell-free supernatant was used for the assay. Antibodies against ovalbumin were raised in rabbits by injecting into multiple sites 1 mg of ovalbumin (99%; Sigma) emulsified in Freund's complete adjuvant. Injections were repeated once a week for 4 weeks and blood was collected 10–14 days after the last injection. Antibodies were purified by $(\text{NH}_4)_2\text{SO}_4$ precipitation (15–40% saturation fraction) and dialysis. Polystyrene wells (7 mm diameter $\times$ 12 mm deep) were coated with anti-ovalbumin immunoglobulin; 300 μl of antibody ($10 \mu\text{g ml}^{-1}$) in 0.2M NaHCO_3 (pH 9.2) was pipetted into each well and incubated at 37 °C for 5 h. Wells were washed three times with wash buffer (PBS containing 0.5% normal rabbit serum and 0.1% bovine serum albumin). In triplicate, cell extract (300 μl) was pipetted into wells and the released antigen was allowed to bind overnight at 4 °C. After incubation, wells were washed three times with wash buffer and 300 μl of ^{125}I -anti-ovalbumin immunoglobulin in wash buffer was added (25 ng ml^{-1} ^{125}I -anti-ovalbumin, specific activity 18 $\mu\text{Ci } \mu\text{g}^{-1}$). Wells were incubated at 37 °C for 3 h, washed three times and individual wells counted in a gamma counter. A standard curve was constructed using ovalbumin diluted in PBS or *E. coli* cell extract. As a measure of the immunoreactive ovalbumin-like material produced in *E. coli* and *M. methylotrophus*, the dilution of each strain giving 50% of the maximum binding was taken to be equivalent to the mass of authentic material giving the same counts. The viable cell count per ml of culture and the mass of ovalbumin detected were used to calculate the number of molecules of ovalbumin-like protein produced per cell. ND, not detected.

demonstrated that these strains are also rendered trimethoprim-resistant (our unpublished results).

Chicken ovalbumin, the major egg white protein, is a polypeptide consisting of 386 amino acids. The DNA sequence corresponding to the entire ovalbumin structural gene has been cloned^{17,18} and ovalbumin-like protein has been detected in bacterial extracts by immunological methods^{19,20}.

A *TaqI* fragment of ~2,200 base pairs, containing the entire ovalbumin structural gene, can be excised from the plasmid pOV230 (ref. 19). The *TaqI* ovalbumin fragment was extended with oligo(dC) tails and annealed to pGSS15 DNA to which oligo(dG) sequences had been added after cleavage with *PstI*. The annealed DNA was used to transform *E. coli* CL499 (*E. coli* CB100 P1 transduced to *rk*⁻*mk*⁺). Ap^rTc^r transformants were screened for the presence of ovalbumin cDNA using a ^{32}P -labelled probe generated by nick-translation²¹ of the *TaqI* ovalbumin fragment. Several positive clones from the hybridization were screened for the production of ovalbumin using an immunoradiometric assay. The plasmid, R751 (ref. 22), was then used to mobilize the hybrid plasmids from ovalbumin-producing *E. coli* strains into *M. methylotrophus*. Table 2 shows the levels of ovalbumin expressed in *E. coli* and *M. methylotrophus*. Restriction enzyme mapping of the inserts of clones which did or did not express ovalbumin confirmed that expression was in one orientation only and was probably initiated at the β -lactamase promoter.

In the plasmid pOMP2 (ref. 20), ovalbumin sequences have been fused to part of the *E. coli* β -galactosidase gene. Expression of this hybrid plasmid yields a product in which the first four amino acids of ovalbumin are replaced by the first eight of β -galactosidase and the system is under the control of the strong *lac* UV5 (ref. 23) promoter. This system leads to high levels of expression in *E. coli* and it was of interest to determine whether similar high levels of expression could be achieved in

M. methylotrophus. The fused β -galactosidase-ovalbumin sequence was isolated from pOMP2 as a single *HhaI* fragment of 2.7 kilobases. pGSS15 was cleaved with *EcoRI* and *PstI* and the large fragment isolated. The *HhaI lac*-ovalbumin and *EcoRI-PstI* pGSS15 fragments were then joined by oligo (dG.dC) tailing. (The construction of these hybrid plasmids is outlined in Fig. 1.) The annealed plasmids were used to transform *E. coli* CL499 and transformants were isolated and analysed in a similar manner to that used for the *TaqI* ovalbumin fragment. Table 2 shows the levels of expression of the *lac*-ovalbumin hybrid sequence in both *E. coli* and *M. methylotrophus*. Restriction enzyme analysis of the plasmid DNA from the hybrids expressing ovalbumin-like protein show that the inserted sequence is expressed in both orientations. However, one orientation (shown in Fig. 1a) is favoured (evident in 9 out of 10 isolates); the significance of this unknown. Expression in both orientations, together with the comparable levels of expression of the *lac*-ovalbumin fragment in pGSS15 and in the parental plasmid is evidence that the expression remains under control of the *lac* UV5 promoter.

The levels of immunoreactive ovalbumin detected in *E. coli* from both the β -lactamase and *lac* promoters are ~50-fold lower than expected. The expression of a penicillinase-proinsulin fused protein produced in similar conditions²⁴ was ~100 molecules per cell and expression of ovalbumin-like protein under *lac* control has been reported at 30,000 molecules per cell^{19,20}, compared with 2.8 and 760 from the present study. The most likely explanation of these differences concerns the assay systems and in particular the specificity, affinity and avidity of the antibodies used. We have tested this hypothesis using a published radioimmunoassay²⁰. The ovalbumin levels detected were similar to those of the immunoradiometric assay described above, indicating that the differences from other published results are probably due to the antisera used.

Although less than in *E. coli*, the level of expression of the two eukaryotic cDNAs in *M. methylotrophus* is significant, indicating that reasonable levels of expression may be achieved in the latter using promoter sequences previously investigated only in *E. coli*. It remains to be determined how closely related these *E. coli* promoter sequences are to the natural *M. methylotrophus* control sequences.

We thank Dr S. N. Cohen for providing pDHFR7, Dr B. W. O'Malley for pOV230, Dr O. Mercereau-Puijalon for pOMP2, Dr B. J. Bachman for CB100 and E. M. Pioli for CL499. We thank Drs M. S. Rose and E. C. Dart and Professor W. J. Brammar for their interest, support and helpful discussions.

Received 30 December 1981; accepted 18 March 1982.

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Evidence that a human β -tubulin pseudogene is derived from its corresponding mRNA

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Pseudogenes—sequences which are homologous to functional genes but which contain mutational changes precluding the formation of a functional product—seem to be a common feature of eukaryotic genomes. Such sequences were first described in the 5S gene of *Xenopus laevis*¹, and have since been found in several gene families including those of the α - and β -globins of several species^{2–10}, immunoglobulin V_H genes¹¹, the actin genes of *Dictyostellum*¹² and the small nuclear RNA genes of man¹³. Among the globin pseudogenes, there appear to be two kinds of structural organization: genes that have retained their intervening sequences^{4–7} and those that have lost them completely^{2,3}. A possible explanation for the generation of such intron-lacking genes involves the reverse transcription of a processed mRNA to form cDNA, and the introduction of this cDNA copy into the genome either by recombinant heteroduplex formation², insertion into a staggered chromosomal break³, or via a retrovirus intermediate¹⁴. Here we report the complete sequence of a human β -tubulin pseudogene. The sequence data reveal the absence of any intervening sequences, and the presence, in the genome, of an uninterrupted 17 base-pair (bp) tract of A residues 14 base-pairs 3' to a poly(A) signal (AATAAA)¹⁵. This sequence organization corresponds closely to the sequence organization of the poly(A) signal and poly(A) tract in a β -tubulin mRNA¹⁶.

Screening of two human recombinant bacteriophage libraries with chicken α - and β -tubulin cDNA probes has resulted in the isolation of most of the tubulin-like sequences contained in the human genome^{17–19}. Structural analysis of the β -tubulin sequences has revealed genes of varying length, including some that are close to, or apparently less than the size required to encode a functional mRNA¹⁷. The complete sequence of one of these genes, determined by the stratagem shown in Fig. 1, is given in Fig. 2. There is a striking overall homology between the chicken and human sequences within most of the coding sequence: 90% of the amino acid sequence is unchanged, while 82% of the bases are conserved. Among those bases that differ, 75% are 'silent' substitutions that do not affect the amino acid sequence. Most of these substitutions are from G or C to A or T residues, accounting for the lower (56%) G + C content of the human relative to the chicken (62%) gene within the coding sequence. The human gene (46 β) contains no intervening sequences. It has a 3' untranslated region which is 44 nucleotides shorter than the corresponding chicken sequence, and is largely non-homologous. Immediately upstream from the initiator ATG in 46 β , the sequence is markedly A + T rich (63%), in contrast to the chicken 5' untranslated region (21%).

Two features render the human sequence incapable of yielding a functional mRNA. First, at amino acid position 230, the serine codon TCG appears as a termination codon (TAA); second, at amino acid position 270, the third base of the phenylalanine codon (TTC) is absent, resulting in a frameshift leading to a termination codon (TGA) beginning at the second

base of amino acid 299. In addition, an unexpected feature of the human sequence is the presence, 14 bp 3' to the poly(A) signal (AATAAA)¹⁵, of an uninterrupted tract of 17 A residues. The spacing between the poly(A) signal and the oligo(A) tract corresponds very closely to the spacing between the chicken cDNA poly(A) signal and its poly(A) sequence. Moreover, the human and chicken sequences share a common trinucleotide (CTT) and pentanucleotide (TTTGT) in this region. This sequence organization strongly suggests that the 46 β human β -tubulin pseudogene arose by reverse transcription from a processed human β -tubulin gene transcript.

A second remarkable feature of the 46 β human pseudogene is the existence of a 11-bp sequence 3' to the poly(A) tract that is directly repeated 170 bp 5' to the initiator ATG (Fig. 2). Because the sequence of a human β -tubulin mRNA has not been determined, it is not possible to identify the equivalent of the cap site in 46 β . However, the location of the repeat sequences is consistent with their generation by repair of a staggered chromosomal break following insertion of the cDNA into the chromosome.

Sequence analysis of a second human β -tubulin gene reveals the occurrence of an in-phase termination codon at amino acid position 361, and the apparent absence of sequences encompassing the entire 5' end up to amino acid position 54 (C.E.C., C.D.W., T.P.C., M.G.-S.L. and N.J.C., unpublished results). In contrast to the data reported here, however, this pseudogene contains a short intervening sequence with correct consensus splice signals. This observation, and the existence of pseudogenes with or without abutting direct repeat sequences¹³, suggests that they were produced by various independent and probably unrelated mechanisms. Gene duplication, with a subsequent loss of selective pressure on one resulting copy, can satisfactorily explain the origin of intron-containing pseudogenes. These sequences could remain linked to their functional counterpart^{4,7–9} or be transferred to a new location by chromosomal translocation, copy insertion or as part of a transposable element. On the other hand, the integration of cDNA copies might occur either directly, or via a retrovirus intermediate, in either case resulting in the random dispersion of intron-lacking pseudogenes throughout the genome.

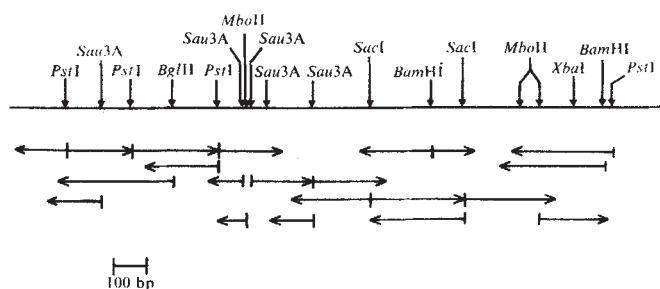


Fig. 1 Sequencing stratagem for clone 46 β . The sequencing method used was that of Sanger *et al.*²⁰. The large *Pst*I fragment was subcloned by digestion of the entire 46 β λ clone¹⁷ with *Pst*I, and ligation of the resulting fragments into pBR322. The appropriate subclone was identified by the method of Grunstein and Hogness²¹ using a chicken β -tubulin cDNA probe²² labelled with ³²P by nick-translation²³. This subclone, or the entire 46 β λ clone, was used as a substrate for digestion with the restriction enzymes shown. In each case, the resulting fragments were subcloned into the replicative form of M13 mp 8 and/or mp 9. Plaques containing DNA to be sequenced were identified on nitrocellulose replicas²⁴ by hybridization with the chicken β -tubulin cDNA probe. Sequence analysis revealed two small *Pst*I fragments containing the 5' end of the 46 β -tubulin gene. These fragments were not detected in previous mapping experiments¹⁷ because of their small size and the limitations of blot transfer procedures.

[illegible]

Fig. 2 Sequence of the human β -tubulin pseudogene contained in clone 46 β . The chicken β -tubulin cDNA sequence¹⁶ is shown for comparison. Homologies with the chicken cDNA sequence were identified using the computer program of Staden²⁵. A termination codon appears at amino acid position 230 (boxed). In addition, a frameshift resulting from a single base deletion at amino acid position 270 has produced an in-phase termination codon (boxed). The poly(A) signals¹⁵ and 3' oligo(A) tracts are underlined. A flanking 11 base-pair direct repeat is overlined. *, Deleted base.

This work was supported by NIH grant GM 26456. C.D.W. acknowledges the award of a Muscular Dystrophy Association postdoctoral fellowship.

Received 27 January; accepted 24 February 1982.

- This work was supported by NIH grant GM 26456. C.D.W. acknowledges the award of a Muscular Dystrophy Association postdoctoral fellowship.
- Received 27 January; accepted 24 February 1982.
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BOOK REVIEWS

The illusory quest for primary colours

J. D. Mollon

THERE is nothing like colour vision for sorting out the men from the boys. Over the past three centuries the field has attracted the strongest minds — Newton, Thomas Young, Helmholtz, Maxwell — and it is an instructive exercise for the modern student to recapitulate the successive insights of these giants. Equally, however, colour vision has always attracted more than its share of heretics and amateurs, perhaps because colour is so immediate a part of everyone's private experience. In his new book P.D. Sherman traces the sequence of development of the trichromatic theory — and describes too the diversionary actions fought by heretics along the way. Finally, however, his own conceptual development does not keep up and, somewhere around the middle of the nineteenth century, he is himself left behind.

The sub-title is a better guide to the book's contents than is the title: the text is almost exclusively confined to trichromatic aspects of colour vision and it covers the period 1700–1860. There is a quite detailed account of the physical trichromatists of the eighteenth century, whereas there is nothing about the developments of the last four decades of the nineteenth century, such as Helmholtz's line-element, Hering's opponent-process theory and König's derivation of fundamental sensitivities that are very close to those of the photoreceptors.

After discussing the historical position of Thomas Young, Dr Sherman devotes a chapter to one of the most influential heretics in the history of colours, David Brewster, who maintained as late as 1850 that there were only three physical forms of light. Dr Sherman describes the experiments that led Brewster to this position and explains how they were finally answered by Helmholtz. He gives a further

Colour Vision in the Nineteenth Century: The Young-Helmholtz-Maxwell Theory. By Paul D. Sherman. Pp.233. ISBN 0-85274-376-9. (Adam Hilger/Heyden: 1981.) £35, \$77.

chapter to one of the neglected heroes of colour science, Grassmann, who introduced the vector representation of colours. Other chapters are devoted to colour blindness and to geometrical representations of colour, the latter illustrated by several colour plates. This period of colour science is covered in greater detail than in any existing text and useful reference lists are provided.

The final two chapters are essentially precis of the great papers on colour vision written by Clerk Maxwell in the period 1855–1860. On the whole the account is admirably accurate and clear (though the student would do as well to read the masterly originals), but when Dr Sherman strays from the path of strict summary he stumbles, and at two crucial points shows that he does not really understand colour vision. The first difficulty centres on the concept of an imaginary primary, a light that has the property of stimulating only a single class of photoreceptors. Since the three classes of receptor have broad and overlapping spectral sensitivities, there do not exist real lights with this property, yet Dr Sherman sometimes suggests that the long-wave fundamental corresponds to an actual wavelength, and he regrets aloud that Maxwell did not measure it. More seriously, he goes on to confuse Maxwell's spectral mixture curves with the actual spectral sensitivities of the receptors. The curves shown by Maxwell represent the proportion of each of his three "standard" wavelengths required to match any other spectral light, but Dr Sherman writes:

"Each curve, being an individual physiological response, shows how each primary stimulates a perceived sensation at each point of the spectrum". In fact, Maxwell's curves are very different from the spectral sensitivities of the photoreceptors; but here Dr Sherman leaves us just as the problem is getting interesting.

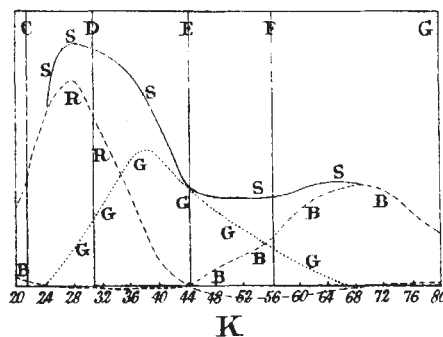
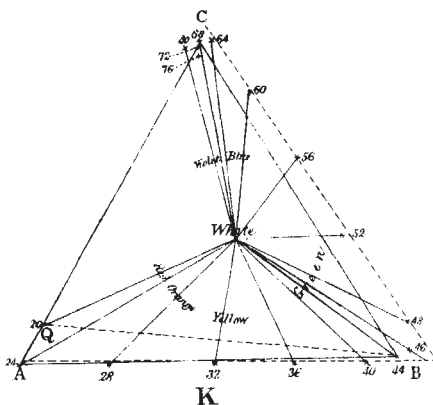
But was Maxwell himself clear on these two related issues? Certainly on the first question he seems twice to have changed his mind. In his first paper on colour (1855) he writes, unambiguously:

Though the homogeneous rays of the prismatic spectrum are absolutely pure in themselves, yet they do not give rise to the "pure sensations" of which we are speaking. Every ray of the spectrum gives rise to all three sensations, though in different proportions . . .

And in his last paper (1871) he writes: "... the true primary red is not exactly represented in colour by any part of the spectrum". In 1860, however, Maxwell tabulated the wavelengths of three "primary colours", adding "I have strong reason to believe that these are the three primary colours corresponding to three modes of sensation in the organ of vision . . ." (*Phil. Trans. R. Soc.*, Vol.150). On the interpretation of spectral mixture curves, his words are more guarded than those of Dr Sherman.

So was Maxwell confused? This is a nice historical question, but I think the answer is probably no, for the following reasons. First, he was working with a version of the trichromatic theory which differs from the modern one: he postulated three primary sensations each corresponding to the activity of an independent class of receptor. We now know that the psychophysical transformation is more

Colour mixing results obtained by Clerk Maxwell for his wife, Katherine. In both diagrams, the numbers 20 to 80 represent arbitrary scale readings on Maxwell's apparatus but correspond to various wavelengths, running from red to blue. By applying a centre-of-gravity rule to the chromaticity diagram on the left, one can find what monochromatic light or spectral mixture will match the colour of a given mixture of three standard wavelengths (say, those wavelengths numbered 24, 44, 68). The 'spectral mixture curves' (R,G,B) on the right show directly the intensities of the three standard colours required to match any given wavelength in the spectrum.



complicated: for example, the "red-sensitive" cones are maximally sensitive in the yellow-green and what is maximal at longer wavelengths is the *ratio* of absorptions in the long- and middle-wavelength cones. Second, given his theory and his actual results, Maxwell had empirical reasons for supposing that there existed three real wavelengths each of which produced a nearly pure sensation; for his results, when plotted in his chromaticity space, suggested that the spectrum locus consisted simply of two straight lines that intersected in the green and thus he was led by his data to suppose that a light placed at any apex of the resulting triangle would come close to stimulating only one sensation.

Maxwell's treatment of colour deserves a more careful assessment than is possible here; but much of the problem has escaped Dr Sherman. The one single error that most held back the development of the trichromatic theory was the belief — still to be heard today — that the peak sensitivities of the photoreceptors must correspond to "primaries", either phenomenologically pure "primary hues" or those primaries in a colour-matching experiment that, by direct mixing, allow the matching of the largest gamut of other colours. Only after

Maxwell was it realized that the latter primaries should be lights that produce the highest ratio of absorptions in different classes of receptor. Dr Sherman's useful book would have been so much the more valuable if it had identified the essential ambiguity of the term "primary colour" in the literature of the mid-nineteenth century. More generally, his readers would have gained if he had much more clearly set out, in an early chapter, the modern distinction between a chromaticity space (representing physical mixtures that produce matches) and spaces in which colours are so placed as to be uniformly spaced phenomenologically. The mapping of one to the other is now known to be very complex.

The historian of science has a peculiarly difficult trade: he must be master of his period and of at least four languages, and he must be also master of the science. If he is to show us the limits of his subjects' thoughts, he must have a conceptual advantage over them and must share this advantage with his reader. He must be Whig without being presentist: the two are not the same. □

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too polished and urbane to harangue us at any time. It is a very scientific book which embodies more neatly than I have seen expressed elsewhere the conviction of most scientists that

... there are many possible worlds; but the interesting one is the world that exists and has already shown itself to be at work for a long time. Science attempts to confront the possible with the actual. It is the means devised to build a representation of the world that comes ever closer to what we call reality. □

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Marine microfossils

B. M. Funnell

Microfossils from Recent and Fossil Shelf Seas. Edited by J. W. Neale and M. D. Brasier. Pp. 380 UK ISBN 0-85312-338-1; US ISBN 0-470-27220-1. (Ellis Horwood/Halsted: 1981.) £35, \$76.65.

A PRINCIPAL merit of this book is the variety of topics it contains. In 28 chapters it covers every geological period from the Silurian to the Recent; a geographical range embracing Spitsbergen and the Barents Sea, East Africa, the equatorial Pacific, the western North Atlantic, Israel, Iran, Pakistan, North-West Europe and the British Isles; and microfossil groups as varied as conodonts, algae, acritarchs, dinoflagellates, ostracods, benthonic and planktonic foraminifers and radiolaria. The organizers of the symposium at Hull University in July 1980, from which this volume derives, were indeed fortunate in having such a wide diversity of contributions on which to draw.

Another successful feature of the book is the high degree of uniformity of contributions, none of which are either too long or too ambitious, and only one of which is reduced to extended abstract dimensions.

The volume as a whole is arranged in stratigraphical order. Approximately 15% is devoted to the Palaeozoic, 35% to the Mesozoic, 20% to the Tertiary, and 30% to the Quaternary and Recent. Most articles apply themselves to stratigraphical or palaeoenvironmental interpretation. No fundamentally new approaches are introduced, and the methodologies used are essentially those developed in the 1960s and early 1970s. A particularly disappointing aspect is the poor understanding of physical and chemical oceanography exhibited by some authors. A few contributions begin to break through this deficiency, for example that dealing with anoxic events in the late Cretaceous and the salutary study of microfossil transport in a modern estuary;

Francois Jacob on possible worlds

P.B. Medawar

The Possible and the Actual. By François Jacob. Pp. 70. Hbk ISBN 0-295-95888-X; pbk ISBN 0-394-70671-4. (University of Washington Press/Pantheon: 1982.) Hbk \$8.95; pbk \$3.95.

THE antithesis embodied in the title of Jacob's short, brilliant and beautifully written little essay can be taken in two ways. In *The Nature of the Physical World*, Sir Arthur Eddington wrote:

I need scarcely add that the contemplation in natural science of a wider domain than the actual leads to a far better understanding of the actual.

An important fraction of Jacob's essay is indeed an exercise of just this kind. It is an exercise more easily possible in the biological than in the physical sciences because in the latter, as Jacob remarks in a Kantian aside, the right-seemingness and naturalness of the natural world may to some extent reflect "the very way our brains work". In biology, however, it is not a tremendous feat to imagine things otherwise than they really are. Why sex, for example? — and why two sexes and not three? The causal-explanatory answer is not easy but the teleological answer that sexuality is "a diversity-generating device" is satisfying and makes sense of what would otherwise be a puzzle.

The second sense in which we may read

Jacob's antithesis is that in which all scientific enquiry is a duologue between the possible and the actual, between what *might* be true and what is in fact the case.

This conception, in which I myself am a firm believer, is sufficiently permissive to make room for myth as an important element in man's attempt to explain his condition, for "whether mythic or scientific, the view of the world that man builds is always largely a product of his imagination". Reflection on this theme allows Jacob to divagate interestingly and amusingly on myths that bear upon sexuality. But of course myths, however effectively they may appease curiosity or dispel the disquiet of ignorance, always fail that difficult exam in which our imaginings are measured up against reality. Yet even in the most "scientific" explanations today there is just a faint hint of the explanatory glibness of the myth — and it is for this reason, I think, that Jacob is a little reserved about natural selection: he does not believe in the universal explanatory value of any one formula.

Needless to say, no one with any understanding of the intellectual tradition to which Jacob belongs and of which he is so able a spokesman could entertain even momentarily the suspicion that Jacob himself is in any serious sense a mystic.

It is an added merit of this book that there is no Message, and that it is altogether

but others, on equatorial Pacific palaeotemperatures and possibly Palaeozoic shelf bathymetries, for instance, tend simply to produce an "acceptable" interpretation that is derivative, and can scarcely be sustained on the microfossil evidence presented.

In spite of these shortcomings the book provides a good review of current research in marine micropalaeontology as practised by members of the British Micropalaeontological Society and their international associates. The inclusion of chapters on the Permian of Iran, the Eocene of Israel and the Recent of East Africa goes a long way

towards redressing what would otherwise be a strong emphasis on temperate zone, North Atlantic and North European occurrences.

This collection of papers should serve a useful purpose in stimulating renewed interest in this currently most useful, but sometimes neglected branch of the earth and biological sciences. It can certainly be recommended for reading by both advanced undergraduates and specialist research workers in the field. □

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Muscle laid bare to the bone

R.M. Simmons

The Structural Basis of Muscular Contraction. By John Squire. Pp.716. ISBN 0-306-40582-2. (Plenum: 1981.) £34.65, \$55.

THE publication of an advanced, or even intermediate, text on muscle is a rare event, an obvious disincentive being that the field is now too big and in many areas too technical to be encompassed satisfactorily in a single book or by a single author. The most one can hope for is an expert treatment of a particular area, and that is what John Squire has done for muscle structure.

Progress in understanding the details of the structure of muscle has depended heavily on the use of X-ray diffraction and on optical diffraction from electron micrographs. The author recognizes the difficulty that diffraction theory presents to non-experts and includes in the introductory material a lucid non-mathematical treatment together with examples of common structures found in proteins and polypeptides. There is also a useful description of the methods used in electron microscopy. Thereafter it is a straight slog through the static structure of muscle, the various isolated and assembled preparations from individual molecules through paracrystals, thick and thin filaments, to superlattices, all 300 pages of it. Such an account could easily have ended up as no more than a useful compendium of techniques and results for the expert, but Squire succeeds on the whole in pursuing what is relevant to understanding structure and function in real muscles. Some readers may find that the author's enthusiasm for research on thick filament assembly exceeds their own, and that in the same area he is a little over-protective towards his own models; there is more than a hint of special pleading for smooth muscle myosin ribbons and the boot goes in a bit heavily into the opposition on vertebrate muscle filaments.

The general outlines of most of the static structures emerge with some clarity. But no question is settled until it is settled right, and that does not come easily in muscle

research. In spite of years of patient grind we still do not know the precise arrangement of the cross-bridges on the outside of the thick filament in relaxed muscle, let alone the packing of the tail portions in the backbone. Doubts have been raised about the steric blocking mechanism of tropomyosin on the thin filament in the regulation of contraction. There are still arguments about the interpretation of the clearest static picture of the intact lattice — insect fibrillar muscle in rigor. The author gives grounds for believing that many of the static structural problems will be solved and indeed there is some confirmation for this view from results too recent to have been included in the book, such as new reconstructions of decorated actin filaments and improved staining methods for cross-bridges in isolated thick filaments.

When it comes to solving the main problem in muscular contraction, of what are the dynamic structural changes underlying force generation and work production, and of how these are related to biochemical events, the grounds for optimism are fewer (and indeed there are fewer pages on it in the book). The author's profession of faith as a molecular biophysicist is that the more one knows about a structure, the more one grows capable of saying how it works. But it is not at all clear that a detailed knowledge of static structure of the filaments or even of the arrangement of cross-bridges in the relaxed and rigor states is of much help in understanding dynamic changes. Contracting muscle displays a high degree of disorder and although improvements in techniques such as time-resolved X-ray diffraction have led to some suggestions about cross-bridge action, there is not much structural information to go on. One has to conclude that a satisfactory solution lies some way ahead and that new techniques will be needed to find it. □

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NEW

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EARL L. GREEN

Director Emeritus of The Jackson Laboratory, Bar Harbor, Maine.
Editor, *Biology of the Laboratory Mouse*, Second Edition.

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